

**Evaluation of water hyacinth (*Eichhornia crassipes*) suitability as feedstock for biogas
production**

By

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DEDICATION

This work is dedicated to my husband Thulani Rich Nkuna and my late granny Rosina Makofane.

DECLARATION

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Exact wording of the title of the dissertation as appearing on the copies submitted for examination:

Evaluation of water hyacinth (*Eichhornia crassipes*) suitability as feedstock for biogas production

I declare that the above mentioned dissertation is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.

SIGNATURE

DATE

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- **Roopnarain A, Adeleke R, Makofane R, Obi L, Lubinga M.** (2018). The Potential of Water Hyacinth (*Eichhornia crassipes*) from Hartbeespoort dam in Biogas and Soil Ameliorant Production, as a Solution to Water Weed Challenges. WRC report. Water Research Commission. Pretoria. *In press*

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SUMMARY

The suitability of water hyacinth in biogas production was evaluated as a means of waste management in the interests of sustainable energy production. Batch anaerobic digestion (AD) of water hyacinth was conducted to determine the optimal pre-treatment method for maximum methane production. Physical pre-treatment methods produced a highest cumulative methane of 2.3 L during batch AD. The selected pre-treatment method, hand-cutting, was further evaluated in a semi-continuous AD using both mono- and co-digestion. The emphasis was on identifying microbial communities involved and their response to organic loading rates (OLRs). The Illumina Miseq results proved that bacterial communities were more sensitive to disturbances caused by irregular OLRs as compared to archaeal communities. In addition, the variation in substrate nutrients as a result of mono- and co-digestion of water hyacinth, contributed to variations in the bacterial diversity. For example, *Bacteroides* and *Petrimonas* diversity varied between mono- and co-digestion. Overall, the study verified that water hyacinth is a suitable feedstock for biogas production and the simple pre-treatment methods are recommended. Furthermore, OLRs influenced the microbial community structure and associated biogas yield.

Keywords: Water hyacinth, pre-treatment, anaerobic digestion, organic loading rates

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LIST OF ABBREVIATIONS

AD	Anaerobic digestion
SO ₂	Sulphur dioxide
CO ₂	Carbon dioxide
CH ₄	Methane
H ₂	Dihydrogen
CaCO ₃	Calcium carbonate
H ₂ SO ₄	Sulfuric acid
CSTR	Continuous stirred tank reactor
HRT	Hydraulic retention time
OLR	Organic loading rate
C/N	Carbon to nitrogen ratio
TS	Total solids
VFAs	Volatile fatty acids
VS	Volatile solids
pH	Potential hydrogen
BMP	Biochemical methane potential
HC	Hand cut
H	Homogenise
OD	Oven dried
SD	Sun dried
HCD	Hand cut and decompose
rpm	Rates per minutes
DNA	Deoxyribonucleic acid
FTTHFS	Formylterahydrofolate synthetase
mcrA	Methylcoenzyme M reductase

ACAS	Acetyl-coA synthetase
PCR	Polymerase chain reaction
OTU	Operation taxonomic units

CHAPTER 1

INTRODUCTION

1.1. Background

One of the major challenges in polluted water bodies to date has been the invasion by water hyacinth, a problematic aquatic plant that is difficult to eliminate once established (Coetzee & Hill, 2012). Although there are various methods of control, the success of these methods is site specific and may be hindered by a number of environmental conditions such as temperature and eutrophication (Hill & Olckers, 2000; Malik, 2007; Moran, 2006;). The ongoing failure of the available control methods for the removal of water hyacinth in invaded water bodies highlights the necessity for sustainable control methods with associated environmental benefits (Kunatsa et al., 2013; Singhal & Rai, 2003; Vaidyanathan et al., 1985; Wang & Calderon, 2012). The use of water hyacinth for biogas production has the potential to be used as an alternative control method that is environmentally friendly. In the past years, the anaerobic digestion (AD) of water hyacinth has been tested and confirmed to have biogas-producing potential (Almoustapha et al., 2009; Kivaisi & Mtila, 1998; Malik, 2007; Ofoefule et al., 2009; Patil et al., 2014; Patil et al., 2012; Patil et al., 2011; Wang & Calderon, 2012).

The biogas produced is derived from the activity of a consortium of facultative and obligate anaerobes, synergistically decomposing organic matter from water hyacinth (Bryant, 1979; Treu et al., 2016). The efficiency of the AD process is dependent on the metabolic activity of microbial consortia which in turn is dependent on a number of controlled and uncontrolled conditions (Chen et al., 2014; Chuang et al., 2011; Liu et al., 2014; Weiland, 2010). The optimisation of water hyacinth AD is necessary due to the previously reported low biogas conversion efficiency when using the plant as a feed (Malik, 2007). Pre-treatment methods that modify the lignocellulosic structure, making organic matter accessible to microbial communities, have been used to optimise the process (Harun et al., 2011; Kurniawan et al., 2014; Ofoefule et al., 2009; Patil et al., 2011). Water hyacinth, like any lignocellulosic biomass, contains lignin as a protective barrier which prevents the biodegradation of cellulose and hemicellulose (Agbor et al., 2011; Hendriks & Zeeman, 2009; Sánchez, 2009). Biodegradation refers to the ability of the compound in question to be decomposed by the action of microorganisms (Angelidaki & Sanders, 2004). Pre-treatment for lignin modification is essential to increase the accessibility of biodegradable biopolymers to microbial communities (Alvira et al., 2010; Demirbas, 2007).

Pre-treatment processes are conducted primarily to accelerate the hydrolysis step (rate limiting step). Hydrolytic enzymes released by microorganisms with the ability to produce extracellular

enzymes are responsible for this stage of AD. Therefore, the rate of hydrolysis is dependent on the activity of the hydrolytic enzymes which in turn are influenced by the increased substrate surface area made available by the pre-treatment methods (Figure 1.1) (Ariunbaatar et al., 2014). In order to evaluate whether the pre-treatment is effective and does not result in the production of inhibitory products, biochemical methane potential (BMP) tests are carried out. A key feature in the selection of the pre-treatment method is the environmental impacts of such methods (Ariunbaatar et al., 2014). For example, in chemical pre-treatment, chemical residues may remain in the digestate, leading to additional challenges in the disposal or downstream application of the digestate. Hence, chemical pre-treatment was not tested in this study.

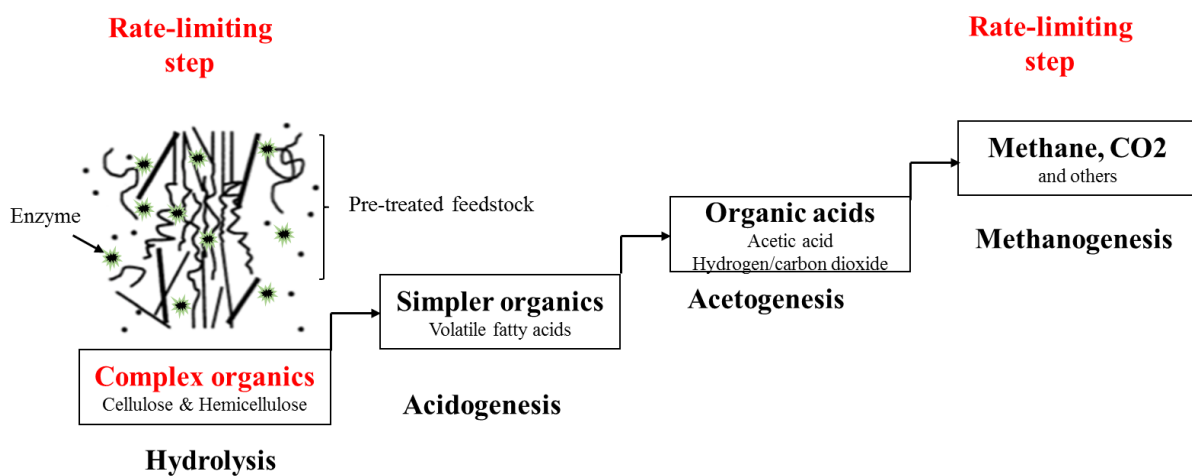


Figure 1.1: Overall AD process (four stages) with the emphasis on the hydrolysis stage

In addition, substrate characteristics are known to influence the type of microbial community present during AD (Ziganshin et al., 2013). In general, the biodegradability of any organic matter is related to its nutrient composition (Amon et al., 2007; Gunaseelan, 2007). Biogas yield depends on both the nutrient availability and the composition of the feedstock which in turn influences the microbial community structure (Amon et al., 2007). Therefore, it is essential to determine the composition and characteristics of the substrate before AD as this allows for an informed, optimisation decision such as the co-digestion of water hyacinth with other sources of organic matter (Kumar, 2005; Malik, 2007; Ofoefule et al., 2009; Patil et al., 2014; Patil et al., 2012). Co-digestion is advantageous because a variation in the nutrients from different substrates improves the carbon-nitrogen (C/N) ratio and buffering capacity (Wang et al., 2014).

Moreover, due to compositional variation, the organic biodegradation capacity of microbial communities will differ, thus making the selection of the organic loading rates (OLRs)

important (Chen et al., 2014; Rincón et al., 2008). Organic loading rate is defined as the measure of the quantity of organic matter fed into the digester per unit volume of the digester (Chen et al., 2014). The lower the OLR the lower the productivity of biogas. However, in instances where the OLR is too high organic overloading is experienced. Organic overloading happens when the volume of organics added exceeds the degradation capacity of the microbes in the digester, thus resulting in process instability (Chen et al., 2014).

In addition, disturbances of the microbial communities occur when there is a sudden change in the OLRs (Chen et al., 2014; Rincón et al., 2008). Theoretically, the OLRs should be consistent to avoid disturbances of the microbial communities and their activity as well as process instability. However, in practice, especially for digesters installed in communities, OLRs consistency may be difficult to maintain. In most situations, this may be due to substrate unavailability, for example, water hyacinth population decreases in winter (Chen et al., 2014). In view of the fact that microbial communities play a key role in biogas production, it is imperative that their activity is maintained. It is, therefore, important to understand both the diversity of microbial divers of the AD process when water hyacinth is used as feedstock and how substrate characteristics influence their selection. This will aid in maximising the efficiency of the process as well as enabling the sustainability of AD as a control method for water hyacinth.

1.2. Rationale

The use of the water hyacinth as a source of biomass for renewable energy production has previously been evaluated (Almoustapha et al., 2009; Kivaisi & Mtila, 1998; Kunatsa et al., 2013; Kurniawan et al., 2014; Lay et al., 2016; Njogu et al., 2015; Malik, 2007), primarily because water hyacinth is a problematic aquatic plant that is difficult to eliminate once established (Coetzee & Hill, 2012). Water hyacinth is characterised by high proliferation rates that enable the plant to cover large areas of the water within a short period of time (Malik, 2007). However, when viewed as a source of biomass for AD, this is an advantage because it relates to the availability and sustainability of water hyacinth as a feedstock for biogas production (Yi et al., 2014).

Currently, the focus is on the optimisation of the AD process for enhanced biogas production and ultimately, the sustainability of the process as an alternative water hyacinth control method. The characteristics of water hyacinth biomass, such as low lignin content, are also advantageous because simple, inexpensive and environmentally friendly pre-treatment such as

size reduction may be used for enhancing the process rather than chemical pre-treatment methods (Ofoefule et al., 2009; Patil et al., 2011). In addition to simple pre-treatment methods, the AD of water hyacinth requires low water usage because the plant comprises 94-95% water content (Ganesh et al., 2005).

1.3. Justification

The current study focuses on a South African dam named Hartbeespoort, located in the North West province. The dam is a 20 km² man-made water storage located 35 km west of Pretoria. It is surrounded by urbanisation, industrialisation, agricultural and mining activities. The dam is regarded as one of the economic hubs for the North West province. However, the water hyacinth invasion of the dam creates a number of problems that threaten the socio-economic development of the dam. Control methods are available (physical, chemical and biological) and all the three available control methods were implemented, with no change or significant control of the plant. In addition, problems such as high cost as well as detrimental effects of chemical herbicides on water quality and no-target organisms in the dam were encountered. Hence, there is a need to find a sustainable use for the water hyacinth that is environmentally friendly and cost effective. An example of such is to use water hyacinth from the Hartbeespoort dam as a feedstock for biogas production.

The use of water hyacinth as a feed for anaerobic digestion will benefit the environment in three ways namely:

- The clean-up of the Hartbeespoort dam as a means of waste management during the physical removal/harvesting of the plant.
- The conversion of water hyacinth through AD to biogas will also benefit the environment. The importance of this process is emphasised due to the prevailing energy crisis, especially in South Africa, and the environmental impacts of the non-renewable energy sources that are predominantly in use. The process of AD reduces the emission of potent greenhouse gases (GHGs), such as carbon dioxide (CO₂) and methane, to the atmosphere as compared to the extraction and production of energy from fossil fuel. The biogas produced may be directly combusted and used for cooking and heat or it may be cleaned and used as automotive fuel and/or electricity.
- The soil ameliorant characteristic of digestate (by product from the AD process) is also beneficial for agricultural application. The substrate that is used for AD consists of both organic and inorganic matter with the microorganisms breaking down mainly the

organic matter during AD, while the inorganic matter is unaffected. The unaffected inorganic matter contributes to the soil ameliorant characteristic of the digestate. In addition, the microbes in AD convert gaseous nitrogen into ammonia (Njogu et al., 2015). Ammonia is a form of inorganic nitrogen, which is available for plant use. As a result of the presence of nutrients in the form that is available for plant use, the digestate has the potential as a soil ameliorant for agricultural purposes.

1.4. Aims and objectives

The study aimed to:

- Decipher the biotechnological processes that may improve the suitability of water hyacinth collected from the Hartbeespoort Dam for biogas production with specific focus on pre-treatment of the plant and the effects of organic loading rates on microbial drivers of the AD process.

Specific objectives

- To determine the chemical composition of water hyacinth collected from Hartbeespoort Dam
- To evaluate the effect of various water hyacinth pre-treatment methods on the biochemical methane potential using batch digesters
- To use the optimal pre-treatment method for biogas production during semi-continuous mono- and co-digestion
- To determine the effect of organic loading rates on microbial composition at various stages of semi-continuous anaerobic digestion.

1.5. Hypothesis

H01: Water hyacinth from Hartbeespoort Dam is a suitable feedstock for biogas production

H02: Biogas production ability of digested water hyacinth is sensitive to irregular changes in organic loading rate

CHAPTER 2

LITERATURE REVIEW

2.1. Water as a limiting resource

Water is a very important resource upon which every living organism depends (Newete & Byrne, 2016). Approximately 70% of the planet is covered by water, however, less than 1% comprises fresh water available for human use (Postel et al., 1996). In South Africa, freshwater sources include rivers, streams, dams and ground water systems (Oberholster & Ashton, 2008). Lakes also are a source of water however, they are located primarily in rural areas and are unsuitable for water supply uses (Oberholster & Ashton, 2008). On the other hand, dams are the most important water source because they serve as water reservoirs that may guarantee the supply of water, depending on the availability of water from the rivers supplying the dams (Oberholster & Ashton, 2008).

In South Africa water is regarded as a limited resource and, thus, dams have been built for the purposes of water storage (Oberholster & Ashton, 2008). The main reason for building dams is to reduce the speed of the flowing water in rivers, thus creating slow-moving water bodies that may be used for irrigation and power generation. (Coetzee & Hill, 2012). *The Annual National State of the Water Report for the Hydrological Year 2012/2013* (Department of Water Affairs) highlighted that South Africa is dependent on the water stored in dams for socio-economic purposes.

Owing to the importance of dams and the potential use of water they contain, the quality of stored water is very important. Stored water is used for human and animal consumption, agricultural and industrial purposes, as well as for maintaining the ecosystem (Matthews & Bernard, 2015). According to Matthews and Bernard (2015), water quality is defined in terms of its chemical and biological composition as well as its physical condition. Thus, water quality depends on a number of factors relating to the intended use. For example, the quality of water used for irrigation will differ from the quality of water used for human consumption. Although dams are important in storing water, the stored water remains in the same place for extended periods, which creates problems in terms of maintaining water quality due to pollution (Oberholster & Ashton, 2008).

2.1.1 Eutrophication

In South Africa, dams that are big enough to supply water for different purposes are located in major metropolitan regions (Oberholster & Ashton, 2008). This serves as an advantage in terms of water supply in the metropolitan regions. However, the high population density in these regions results in large amounts of waste generation with concomitant effects on the water

quality in the dams. Apart from being surrounded by residential areas, dams are also often surrounded by industrial, agricultural and mining activities. Examples of such dams include the Hartbeespoort Dam in the North West Province and the Vaal Dam in Gauteng province. Such surrounding environments have a negative effect on the quality of water stored in dams

Pollution in water bodies is caused by an increased concentration of nutrients – this process is known as eutrophication (van Ginkel, 2011). Surrounding environments are the main contributors of pollution in the dams through the deposition of nutrients, mainly nitrogen and phosphorus, in the water (Oberholster & Ashton, 2008). Although eutrophication occurs naturally during the ageing process of lakes or dams, the surrounding environments may accelerate the process (Fox et al., 2008). Water quality is classified according to trophic levels to monitor the nutrient status. Trophic levels refer to the amount of nutrients in water as described in Table 2.1 (Matthews & Bernard, 2015).

Many of the fresh water reservoirs in South Africa have been subjected to degradation through pollution and a number of them are already in a state of eutrophication or hypertrophication. Figure 2.1A depicts the trophic status and eutrophication potential of some of South Africa's water bodies. Eutrophication potential is described as the likelihood for future problems based on the phosphorus concentration (van Ginkel, 2011). The pollution of stored water contributes to limited water usage as well as a number of deleterious effects that further degrade water quality.

Table 2.1: Classification of trophic levels (Adapted from Water Quality Report No: PWMA 01/B50/00/8310/7).

State	Description
Oligotrophic	Low in nutrients
Mesotrophic	Intermediate levels of nutrients
Eutrophic	Rich in nutrients
Hypertrophic	Very high nutrient concentrations

2.1.2 Disadvantages of eutrophication

In addition to affecting the usage of water, eutrophication is also viewed as a threat to aquatic ecosystems. Nutrient enrichment in water bodies favours the growth of aquatic organisms that prefer high nutrient concentration, thus changing the biodiversity of the water bodies. Eutrophication is usually associated with the excessive growth of waterweeds such as

macrophytes (van Ginkel, 2011), which are defined as unwanted aquatic plants that are problematic once established in water bodies (Deivasigamani, 2013). Examples of such include water hyacinth (*Eichhornia crassipes* (Mart.) Solms), water lettuce (*Pistia stratiotes*), red water fern (*Azolla filiculoides*), water fern (*Salvinia molesta*), parrot's feather (*Myriophyllum aquaticum*), the bullrush (*Thypha capensis*) and the reed (*Arundo donax*) (Cilliers et al., 2003; Jones, 2001).

Water hyacinth has been highlighted as the most problematic and damaging of all the invasive aquatic plants listed (Cilliers et al., 2003). It is described as an aggressive, free-floating, aquatic species which is notorious for its rapid reproduction (Malik, 2007). It belongs to the family pickerelweed (Pontederiaceae) (Kunatsa et al., 2013). The plant comprises dark green, thick, glossy round leaves attached to spongy petioles containing air-filled sacs to enable the plant to float in water (Patil et al., 2011; Sudani et al., 2014). The plant's growth may vary from few centimetres to 1 meter depending on the environmental conditions (Kunatsa et al., 2013). It is indigenous to South America but is currently found in many lakes, dams, rivers and swamps in tropical and subtropical countries (Kunatsa et al., 2013).

Water hyacinth can successfully outcompete other aquatic plants and tolerates a wide range of environmental conditions although it prefers nutrient-rich environments (Malik, 2007). The plant reproduces sexually or asexually (Buchanan, 2014). The sexual reproduction is rare and results in the production of seed in which their germination may occur in a few days or may remain dormant for years (Malik, 2007). The asexual reproduction is common and occurs by budding or stolon reproduction. This type is associated with rapid reproduction resulting in the ability of the plant to double its population within a week. The plant's growth is directly correlated to the nutrient concentration in the water bodies, especially the nitrogen and phosphorus levels (Heard & Winterton, 2000).

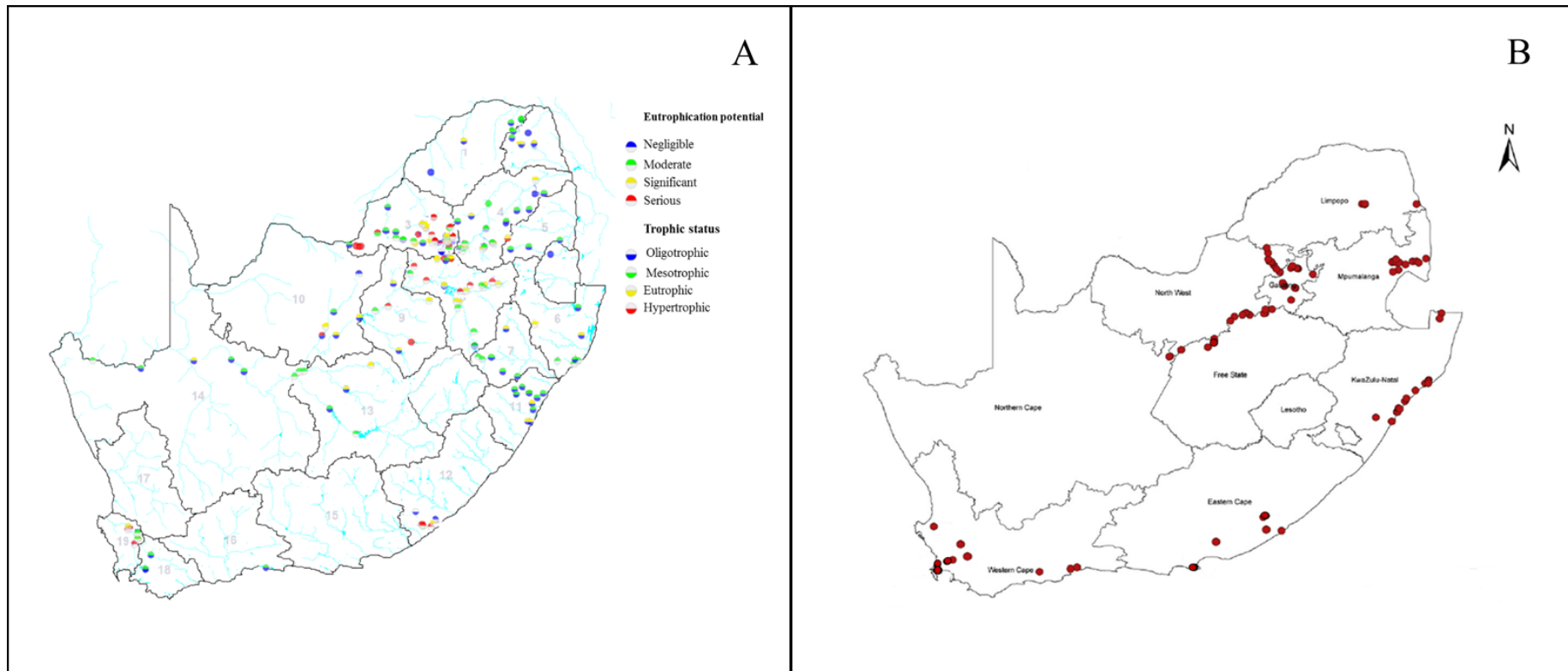


Figure 2.1: Map of South Africa showing the trophic status of dams and water hyacinth invasion. A – Eutrophication potential and trophic status of dams and rivers in South Africa (adapted from *the Annual National State Water Report for the Hydrological Year 2012/2013*), B – Distribution of water hyacinth in South Africa (Adapted from Albano et al., 2011)

2.2. Water hyacinth in South Africa

South Africa is one of the countries that has suffered from the invasion of water hyacinth in many of its aquatic ecosystems. The main reason for this is that South Africa has the most eutrophic aquatic ecosystems in the world (Coetzee & Hill, 2012). It is possible that the eutrophication problem may be related to the 1 mg/l of phosphorus standard which has been adopted for all water treatment by the South African Department of Water Affairs (DWAF). In addition, the dams also create favourable growth conditions for water hyacinth due to the slow-moving water (Coetzee & Hill, 2012). Water hyacinth is widely distributed in aquatic ecosystems throughout South Africa (Figure 2.1B). Dams such as Hartbeespoort (North West province), Roodeplaat (Gauteng province) and Kleinfontein (Benoni, Gauteng) and rivers such as the Vaal (Gauteng province) are all examples of water bodies with severe water hyacinth invasion in South Africa.

The existing literature suggests that the significance of the invasion depends primarily on the trophic status of the water body (Coetzee & Hill, 2012). Accordingly, the eutrophic state of the water is the main reason for the high proliferation rate of water hyacinth in comparison to water in the oligotrophic and mesotrophic states. This is further illustrated in Figure 2.1A and Figure 2.1B, which show that the distribution of the water hyacinth is concentrated mainly in water bodies that are in the eutrophic and hypertrophic states. A study by Heard and Winterton (2000) measured the growth of water hyacinth using high and medium nutrient concentrations (0.4mg/l-1.6 mg/l nitrogen and 0.025 mg/l- 1 mg/l phosphorus). Their results showed that, at high nutrient concentration, the plant multiplied quickly, thus increasing the biomass and, therefore, supporting the notion that nutrient-rich water bodies increase the chance of water hyacinth invasion.

2.2.1 Problems related to water hyacinth invasions

Water hyacinth is characterised by high proliferation rates in nutrient-rich water bodies (Deivasigamani, 2013). This enables the plant to cover water surfaces in a short period of time (Yan et al., 2017). The high proliferation rate has become a large threat to socio-economic development (Cilliers et al., 2003). This is because the dense mats that the water hyacinth produce cause degradation of water quality and consequently limits water utilisation (Shanab et al., 2010). Eutrophication and the absence of natural enemies of this plant also contribute to the high growth rates (Charudattan et al., 1995).

The plant degrades water by blocking the light penetration into water bodies for other submerged aquatic plants, thereby decreasing the dissolved oxygen (affecting fish population), inducing higher evapotranspiration and preventing water activities (Gupta et al., 2012; Villamagna & Murphy, 2010). Dense mats of the plant also create a breeding habitat for malaria, encephalitis and filariasis carrying mosquitos as well as the snails which are a vector for schistosomiasis (Bhattacharya et al., 2015; Malik, 2007). Villamagna and Murphy (2010) explain that water hyacinth mats provide a habitat for aquatic invertebrates, thus increasing the population and diversity of water birds. These organisms alter the ecosystem and disrupt both the nutrient cycling and the food chain (Shanab et al., 2010).

Human activities are the major cause of the spread of the plant to other water bodies, although moving water in streams and wind also contribute to the spread (Kato-Noguchi et al., 2014). As mentioned in section 2.1, dams create very favourable growth conditions for water hyacinth due to the slow-moving water. In other words, stream invasion is usually less severe in comparison to dams. In streams, the water movement breaks the mats while, in dams and lakes, the mats grow and cause the above-mentioned problems.

2.3. Water hyacinth control methods

Water hyacinth is one of the world's invasive aquatic plants and is extremely difficult to eliminate (Heard & Winterton, 2000). Accordingly, the plant was listed by the Conservation of Agricultural Resources (Act 43 of 1983) as a weed (category 1), thus indicating that the invasive plant is prohibited on any land or water surfaces in South Africa and must be controlled or destroyed where possible. Control methods that have been developed for managing the proliferation of the plant include the physical removal of the plant through harvesting, the application of chemicals, such as herbicides (Jones, 2001) and the use of biological agents such as weevils (Coetzee et al., 2007). These methods are elaborated on below.

2.3.1 Physical removal

Physical removal refers to the direct removal or harvesting of the plant from water surfaces, either manually or mechanically (Figure 2.2) (Vásquez et al., 2015). This is usually the first option used to control water hyacinth in most countries in which their water bodies have been invaded (Cilliers et al., 2003). Although considered as an environmentally friendly method (Malik, 2007), the removal of the plant is usually temporary when the plant is removed on a periodic basis. Accordingly, this method is regarded as a short-term control option (Cilliers et

al., 2003). The mechanical harvesting of water hyacinth is expensive, labour intensive and time-consuming (Deivasigamani, 2013) and, thus, the method is not sustainable as a short-term control option. As an example, the eutrophic Isiphingo River (eThekweni municipality, South Africa), which was covered by water hyacinth, was treated using the physical harvesting control method. However, soon after clearing the river, the water hyacinth regenerated faster than before and covered the water surface with denser mats, worse than before the harvesting (Environmental Planning and Climate Protection Departments, eThekweni Municipality, 2001). The harvesting of the plant is effective if it is continuously removed and not just periodically.



Figure 2.2: Physical removal of water hyacinth from the Hartbeespoort Dam in North West Province, South Africa (https://www.youtube.com/watch?v=_80Mkmtb9I)

2.3.2 Use of chemical herbicides

The second control option is the use of chemical herbicides. African countries such as South Africa, Ghana, Nigeria, Zambia and Zimbabwe have all been involved in the use of chemical herbicides to control water hyacinth invasion. When compared to the physical approach, the chemical approach has attracted more attention due to the shorter duration of the time required to implement chemical control methods (Deivasigamani, 2013). However, the use of chemical herbicides has high cost implications. Furthermore, apart from controlling the target plant, the chemicals further degrade the quality of water and inhibit the growth of other aquatic organisms. In particular, the environmental impact of the herbicides may last for years. A good

example of this is the Hartbeespoort Dam, South Africa. The Department of Water Affairs decided to use chemical herbicides to control the spread of the plant in the late seventies (Oberholster & Ashton, 2008). However, several years later the infestation of water hyacinth in the dam has become even worse.

2.3.3 Biological control

Biological control is considered to be the only sustainable method for controlling the plant (Cilliers et al., 2003). Owing to the cost implications of the control of the waterweed using the physical and the chemical methods, the biological control method has been embraced. This method reduces the cost implications by using natural enemies, such as insects, which kill or reduce the population of water hyacinth to below the level of economic damage (Vásquez et al., 2015). It has been established that there are approximately six insects that are known to be the natural enemies of water hyacinth. These include *Neochetina eichhomiae*, *Neochetina buchi*, *Eccritotarsus cetarinensis*, *Niphographa albigutalis* and *Othogalumna terebrantis* (Hill & Olckers, 2000). However, the biological control method requires a minimum of three to five years to be effective. This is because insects are used and, thus, their population needs to increase to a level where it will cause a significant reduction in the plant population (Deivasigamani, 2013).

South Africa has already released all six of these natural enemies into the various aquatic ecosystems invaded by the plant (Hill & Olckers, 2000). The success of this control method has been seen in other countries (Lake Victoria) where very dense mats of water hyacinth were significantly reduced by the attack of the natural enemies of the plant (Cilliers et al., 2003). However, biological control methods have been less successful in eutrophic water bodies in areas with favourable growth conditions for water hyacinth such as temperature (Coetzee & Hill, 2012). Although this method is efficient, the environmental impacts of the biological agents once the water hyacinth has been significantly reduced, are still being researched (Malik, 2007).

2.3.4 Failure of control methods

Globally, despite the available control options, water hyacinth remains the most problematic waterweed in countries facing this challenge (Coetzee & Hill, 2012; Heard & Winterton, 2000). The control methods are usually hindered by a number of factors such as temperature, trophic state and size of the aquatic ecosystem, hence, the success of the control methods may be site-specific (Hill & Olckers, 2000; Malik, 2007; Moran, 2006). In South Africa, the control of the

plant is an issue of major concern due to the ability of the plant to further degrade the water quality of the rivers and dams or catchments (Coetzee & Hill, 2012). However, it is pertinent to mention that there have been a number of successful reports on the use of the three control methods – the physical, chemical and biological – in South Africa (Hill & Olckers, 2000), such as the Mposa River (uThungulu District Municipality). This was one of the rivers heavily invaded by water hyacinth and as a result, all the three control methods were used. An integrated water hyacinth control programme was introduced which resulted in the reduction of water hyacinth infestation to an acceptable level. Accordingly, Jones (2001), stated that the water hyacinth infestation remained within acceptable level from 1995, when the programme was introduced, to 2001. However, occasional follow-ups to spray with herbicides or physical removal of water hyacinth were required.

Coetzee and Hill, (2012) highlighted that the success of water hyacinth biological control will be inhibited by a high nutrient concentration in water bodies because the plant will be able to tolerate and recover quickly from insect attack. Eutrophication is a problem in South Africa, with the presence of water hyacinth further degrading water quality. Water is very important and it is imperative that the degradation of water quality is avoided or managed to ensure water conservation (Newete & Byrne, 2016). It is, therefore, vital that the sustainable potential uses of water hyacinth to enable biomass management are implemented.

2.4. Water hyacinth as a resource

The biological characteristics of water hyacinth pose a number of challenges and opportunities to the researchers in countries that have been invaded by the plant. Yan et al. (2017) described the biological characteristics of the water hyacinth as ‘unique’ due to the number of capabilities of the plant. Although the plant has a number of negative environmental effects, research efforts have proven that the characteristics of water hyacinth also have the potential to outweigh the problems the plant causes thus, making it an important resource in the future (Okoye et al., 2002; Malik, 2007; Patil et al., 2014; Sanni & Adesina, 2012; Wang & Calderon, 2012; Yan et al., 2017). Examples of such potential uses include anaerobic digestion of the weed to different end products such as biogas, alcohol, and bio-fertiliser. In addition, it has been found useful in phytoremediation, production of compost, animal fodder, furniture and ropes (Malik, 2007).

However, the major setback with most of these uses was the lack of sustainable economic use (Malik, 2007). In many instances, some of the potential uses did not succeed in controlling the plant. However, current advances in research are focusing not only on finding a sustainable

economic use but also exploring the potential advantages that the plant has to offer to the environment (Hussain et al., 2010; Shanab et al., 2010). An example of such potential advantages includes the use of the water hyacinth plant for phytoremediation and biogas production. The plant is also able to produce allelopathic substances (Kato-Noguchi et al., 2014; Wang & Calderon, 2012).

2.4.1 Benefits of water hyacinth to the environment

2.4.1.1. Allelopathic property

Although water hyacinth is an unwanted plant in water bodies, it does, nevertheless, have a number of characteristics that are beneficial to the environment (Heard & Winterton, 2000). The main feature that allows the plant to outcompete other aquatic plants is its ability to produce allelopathic chemicals (Shanab et al., 2010). Allelopathic chemicals refer to the secondary metabolites produced by the plant, which have the ability to inhibit the growth of other organisms (Kato-Noguchi et al., 2014). Accordingly, the large biomass of the plant corresponds to high allelopathic chemical production which inhibits other invasive aquatic plants, thus explaining the ability of the plant to outcompete other aquatic plants.

In a study by Kato-Noguchi et al. (2014), the allelopathic properties of water hyacinth were tested against a number of aquatic plants. The study found that the extracts from the plant inhibited the growth of the shoots and roots of these aquatic plants. Another study by Shanab et al. (2010) focused on testing allelopathic properties of water hyacinth against a number of microorganisms and found that the Gram-positive bacteria which had been tested were inhibited. The same study found water hyacinth to have antialgal properties and when tested against green microalgae and cyanobacteria, it was found that their growth was inhibited. Therefore, since water hyacinth is not the only problematic species in the aquatic ecosystems responsible for reducing water quality, the ability of the plant to inhibit the growth of other organisms reduces the number of problematic species in water bodies.

2.4.1.2. Phytoremediation ability

The process in which plants are used to remove nutrients from polluted or wastewater is known as phytoremediation (Fox et al., 2008). Water hyacinth plants were found to have the ability to remove nutrients from polluted water bodies (Fox et al., 2008; Gupta et al., 2012; Hussain et al., 2010; Jasrotia et al., 2015). This is particularly important for a country like South Africa where water is scarce.

In its natural habitat, water hyacinth floats on water surface and the roots are submerged in the water where they are exposed to nutrients (Malik, 2007). The plant uses the nutrients for growth while also reducing their concentration in the water (Fox et al., 2008). Thus, the degradation of water quality due to pollution can be reduced by using water hyacinth. A study by Fox et al. (2008), evaluated the ability of water hyacinth in the removal of nitrogen (N) in ponds. Different concentrations of N were used. It was subsequently found that the water hyacinth biomass increased with increasing N concentrations and, although total removal of the N did not occur, the plant accounted for 60 to 85% of the N removed. This process is both environmentally friendly and inexpensive and, in addition, the plant can be adapted to a wide range of environmental factors.

Water hyacinth plant is sometimes referred to as ‘a blessing in disguise’ because it improves the physical appearance of water (Ephraim & Ugbaja, 2018). Thus, water hyacinth has significant potential in phytoremediation. Problems arise when the plant growth is not controlled and where dense mats are formed (Mironga et al., 2014). This results in a cycle that releases the nutrients back into the water when the plant dies, decomposes and sinks in the water (Menon & Holland, 2014). However, if the growth and spread of the plant is controlled, water hyacinth may be useful in remediating water bodies. Effective phytoremediation requires a controlled system, where the plant is harvested to avoid nutrient recycling. Thus, instead of harvesting and allowing the plant to decompose on the shore, the plant may be used as a source of biomass to produce renewable energy.

2.4.1.3. The need for renewable energy production

It is known that fossil fuels such as coal, petroleum oil and natural gas account for over 80% of the world’s primary source of energy, while renewable energy accounts for about 14% only (Moriarty & Honnery, 2012; Song et al., 2012). This is a problem because fossil fuels are associated with a number of environmental challenges such as emission of potent greenhouse gases. Moreover, due to the growing global population, the energy demand is continuously increasing (Song et al., 2012). Although developed and developing countries have increased their energy production processes to satisfy the needs of the populations involved, problems still arise that raise questions about the security of global energy in the future (Saxena et al., 2009).

Fossil fuels are extracted mainly from naturally occurring reserves. However, due to high energy demand, these reserves are diminishing very rapidly, thus increasing energy costs

(Agbor et al., 2011; Budiyo et al., 2010; Ganguly et al., 2012). In addition to the rapid depletion rates of the reserves and the high costs, the extraction, production, transportation and utilisation processes have caused pollution that is resulting in climate change, environmental degradation, and human health problems (Budiyo et al., 2010; Machol & Rizk, 2013).

The extraction of these compounds emits sulphur dioxide (SO_2) to the environment (Saxena et al., 2009). When released into the atmosphere, SO_2 reacts with humidity forming sulphuric and sulphurous aerosol acid – both of which form part of acid rain (Bolzonella et al., 2003). Moreover, the utilisation or burning of these fossil fuels emits carbon dioxide (CO_2), a greenhouse gas known to be the major contributor to climate change (Bolzonella et al., 2003). Accordingly, a number of major problems, including pollution, depletion rates and cost, are associated with fossil fuel usage (Saxena et al., 2009). Thus, there is a definite need for alternative renewable energy sources both to satisfy the demands of energy for the growing population and to reduce the emission of harmful gases into the atmosphere (Budiyo et al., 2010).

Renewable energy is a type of energy that is clean (environmentally friendly) and produced from natural resources. Sources of renewable energy include solar, hydropower, wind, geothermal and biomass energy (in the form of biogas or biofuel) (Song et al., 2012). The utilisation of water hyacinth as a potential source of biomass in renewable energy production has a number of advantages. For example, it offers an alternative source of energy that has the potential of reducing the emission of SO_2 and CO_2 into the environment while it will play a role in cleaning water bodies invaded by the plant (Chaturvedi & Verma, 2013).

2.5. Biogas production

Biogas is a mixture of methane (50 to 70%), carbon dioxide (30 to 40%) and traces of other gases (Patil et al., 2011). It is produced through a complex process called anaerobic digestion (AD). Anaerobic digestion is an important cost-effective technology for recycling organic matter to produce renewable energy (Mata-Álvarez et al., 2000). It is defined as a multi-stage biological process in which any biodegradable organic matter is broken down by microorganisms in the absence of oxygen for energy and growth through different stages to produce biogas and a nutrient-rich digestate (Bryant, 1979; Mukuba et al., 2018; Sahito et al., 2013).

Currently, AD is reputed to be a reliable technology for treating municipal, industrial and agricultural waste (Budiyo et al., 2010). Although AD technology is used for numerous

applications by different sectors, the primary goal of all these sectors is to benefit the environment (Yadvika et al., 2004). The benefits to the environment include the treatment and stabilisation of waste (size reduction) as well as reducing CO₂ emission (Amon et al., 2007; Mata-Álvarez et al., 2000).

Biogas production by AD occurs in four stages, which include hydrolysis, acidogenesis, acetogenesis and methanogenesis (Leung & Wang, 2016). Stages 1-3 mainly represent the conversion of the feedstock into different compounds while the last stage is the stabilisation of the compounds to produce biogas (Figure 2.3) (Leung & Wang, 2016). Microbial communities are the key drivers of AD and each stage is associated with a unique group of microorganisms that differ in nutritional and pH requirements (Ziganshina et al., 2015). Fermentative bacteria and fungi are known to occur during hydrolysis, acidogenesis and acetogenesis while methanogenesis is carried out by archaeal consortia (Ziganshin et al., 2013).

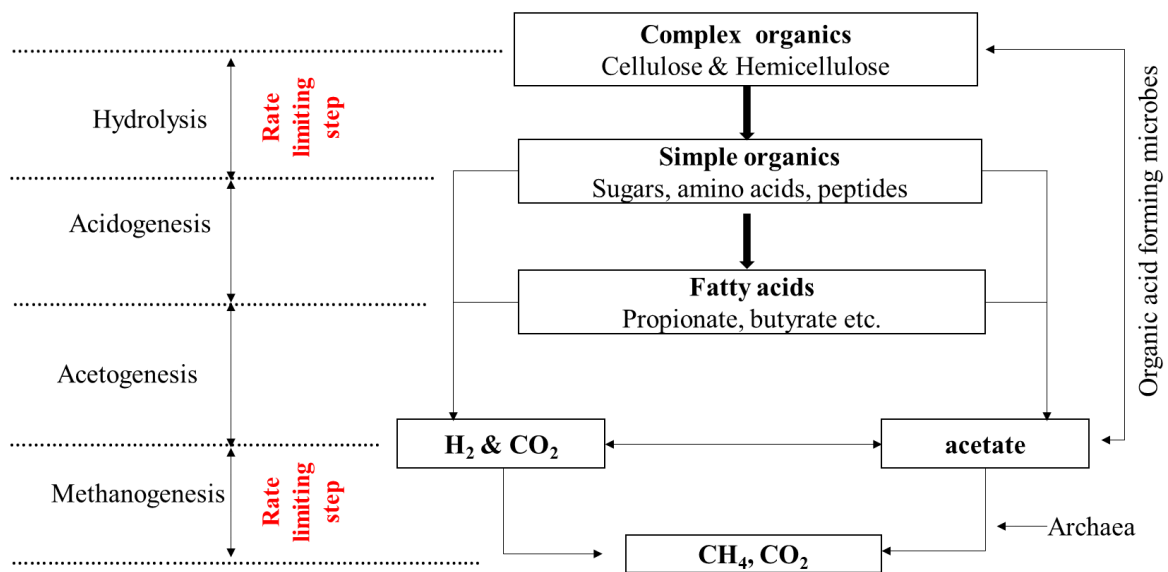


Figure 2.3: Microbial processes involved in anaerobic digestion (adapted from Chen et al., 2016; Molino et al., 2013)

2.5.1 Hydrolysis

Hydrolysis is the first stage of AD in which complex organic matter is broken down by extracellular enzymes secreted by microorganisms (Figure 2.3). They are broken down into simple organics such as sugars, fatty acids and amino acids (Molino et al., 2013; Ziganshin et al., 2013). However, complex organics such as lignocellulose are not always freely accessible from the substrate, making hydrolysis a rate-limiting step.

Lignocellulosic biomass is composed of different biopolymers linked together by different bonds to form a rigid structure, resistant to microbial degradation (Hendriks & Zeeman, 2009). The three biopolymers that form this rigid structure are cellulose, hemicellulose and lignin (Figure 2.4A) (Sánchez, 2009). Cellulose is a major component of the plant cell wall, representing up to 50% of the support structure (Agbor et al., 2011). Hemicellulose is the second biopolymer representing about 20 to 30% of the support structure (Agbor et al., 2011). Lignin is a biopolymer of aromatic compounds and differs from cellulose and hemicellulose in that it is rigid and impermeable, serving as a protective barrier against the microbial degradation of cellulose and hemicellulose (Agbor et al., 2011).

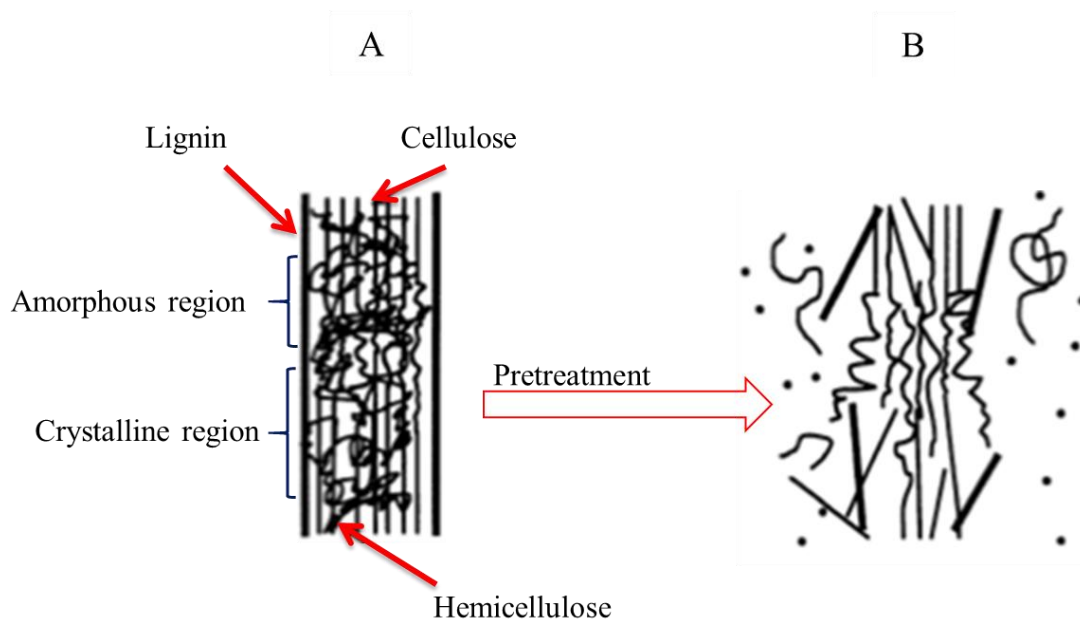


Figure 2.4: Pre-treatment of the lignocellulosic structure (adapted from Harmsen et al., 2010), A - unmodified lignocellulose and B - modified lignocellulose

2.5.1.1. Pre-treatment methods

The presence of unmodified lignocellulose (Figure 2.4A) affects the rate of hydrolysis. In addition, the subsequent AD steps are dependent on the product of this stage and, hence, the AD process as a whole is affected. In order to increase the rate of hydrolysis, the disruption or modification of the protective barrier is required to expose the two biodegradable biopolymers. This is achieved through the pre-treatment of the substrate before AD.

Pre-treatment is a method which is currently available to improve the solubility and bioavailability of organic matter. The process disrupts or modifies the recalcitrant structure to increase the bioavailability of the two important biopolymers (Figure 2.4B) (Alvira et al.,

2010). Pre-treatment methods are categorised into four different groups, namely, physical, chemical, biological and a combination of any of these methods (Harun et al., 2011). Each method uses a different mechanism to increase the accessibility of the organic matter. The physical methods focus on size reduction as well as reducing the degree of polymerisation (Harmsen et al., 2010). While chemical pre-treatment results in the swelling of the biomass, thus breaking the lignin and the carbohydrate linkages (Agbor et al., 2011). The biological pre-treatment uses microorganisms that produce extracellular enzymes or commercially available enzymes to disrupt the lignocellulosic structure (Ariunbaatar et al., 2014).

Theoretically, the purpose of pre-treatment is to open up the cell wall to allow hydrolytic enzymes to break down the molecules (Demirbas, 2007). However, there are variations in the biogas yield for the different pre-treatment methods. An effective pre-treatment method of lignocellulose should have the following properties (Agbor et al., 2011).

- It should preserve and decrystallise the celluloses and depolymerise hemicelluloses.
- It should restrict the formation of inhibitors which negatively affect the hydrolysis of carbohydrates.
- It should prevent sugar degradation.
- It should result in the recovery of most of the usable sugars.
- It should have low energy input as well as being cost-effective.

However, the effect of pre-treatment depends on the substrate characteristics. Variations in the characteristics of the substrates cause them to react differently when pre-treated (Carlsson et al., 2012). Thus, the selection of the pre-treatment method prior to AD is considered a crucial step because some methods may produce compounds that may inhibitory the activity of the key microbial communities in the AD process (Harmsen et al., 2010).

Once lignocellulose modification is achieved, the hydrolytic enzymes that carry out hydrolysis are able to easily convert the biopolymers to fermentable sugars for use in the subsequent stages (Harmsen et al., 2010). Examples of hydrolytic enzymes include protease, lipase and cellulase. In addition, microbial species, such as *Bacteroides succinogenes*, *Ruminococcus flavefaciens*, *Clostridium cellobioporos* and *Clostridium thermocellum*, are known to produce these enzymes during hydrolysis (Bayané & Guiot, 2011).

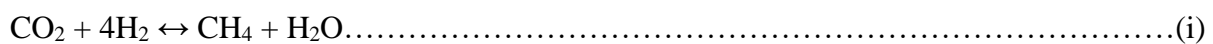
2.5.2 Acidogenesis and acetogenesis

The second stage, acidogenesis, is carried out by acid forming bacteria that convert the simple organics produced during hydrolysis to volatile fatty acids (VFAs), alcohol, ketones, CO₂ and ammonia (NH₃) (Leung & Wang, 2016). The third stage, which is acetogenesis, results in the production of acetic acid, CO₂ and hydrogen (H₂) from the products of acidogenesis. (Yadvika et al., 2004). Acidogenesis and acetogenesis occur rapidly and it is difficult to distinguish between the two stages (Bajpai, 2017). These two stages are carried out by both facultative and obligate anaerobes such as *Clostridium*, *Bifidobacterium*, *Desulfovibrio*, *Lactobacillus*, *Peptococcus*, *Actinomyces*, *Seimonas*, *Staphylococcus*, *Streptococcus*, *Corynebacterium*, *Micrococcus*, *Bacillus*, *Pseudomonas*, *Veillonella*, *Sarcina*, *Desulfobacter*, *Desulfomonas* and *Escherichia coli*. Species dominance is determined by the substrate characteristics (Kosaric & Blaszczyk, 1992).

2.5.3 Methanogenesis

The last stage is known as methanogenesis (Chen et al., 2016) and is carried out mainly by archaea such as *Methanosarcina*, *Methanobacterium*, *Methanobrevibacter*, *Methanothermobacter* and *Methanosaeta*. These microorganisms produce the final products of AD, which is a mixture of methane (CH₄) and CO₂ as well as by-products. Methanogenesis is also a rate-limiting step due to the slow-growing nature of methanogens and their sensitivity to pH (Leitão et al., 2006). Methanogens use the acid compounds produced during stages 2 and 3 but, if the population is unable to utilise the compounds at a fast rate, the acidic compounds will accumulate, thus decreasing the pH and disrupting the activity of the methanogens. Methanogens prefer an optimum pH range of 6.5-7.5 (Yoshida et al., 2008).

There are three types of methanogens, which are differentiated according to the CH₄ producing pathways. However, some of these methanogens are able to produce methane using more than one pathway. Hydrogenotrophic methanogens are known as cytochrome-lacking methanogens and use hydrogen as a source of electrons for reducing CO₂ into CH₄ (i) (i) (Richards et al., 2016). This type of methanogen produces approximately 30% of the total CH₄ during AD. The second type of methanogens, the acetotrophic methanogens, use acetic acid to produce CH₄ and CO₂. They possess cytochrome and are responsible for producing over 70% of the total CH₄ during AD (ii) (Leung & Wang, 2016). The last type, methylotrophic methanogens, uses an uncommon pathway to produce CH₄. This type also possesses cytochrome but CH₄ production occurs only in the presence of a methyl group (iii) (Leung & Wang, 2016).



2.5.4 Selection of microbial community in AD

A number of environmental factors influence the selection of a specific microbial community in AD (Levén et al., 2007). However, the substrate is the major contributor in terms of microbial community structure. Different types of carbon substrate affect the microbial community structure (Ziganshin et al., 2013). In addition, substrates with a high carbon content result in a diverse microbial community.

According to Carballa et al. (2015), the more diverse the microbial community structure, the better the AD performance. This diversity is linked to variation in nutrient composition. Thus, in most AD processes, different substrates are combined (Co-digestion) to create a balance in the final nutrient contents and concentration. Other factors that influence the selection of specific microorganisms in AD include bioreactor design and operating conditions. For example, studies by Leclerc et al. (2004) and McHugh et al. (2004) have shown that variation in bioreactor design, operating conditions and feedstock type all influenced the microbial community structure.

The performance of microbial communities in the AD process depends on the availability of nutrients from the substrate (Bryant, 1979). The nutrients required by these microorganisms are divided into macronutrients (nutrients that are required in high concentration) and micronutrients (nutrients required in low concentration). Any limitation of these nutrients contributes to process failure (Demirel & Scherer, 2008; Liu et al., 2014).

Examples of macronutrients include carbohydrates, fats, proteins, potassium, magnesium and calcium. These have different functions and ensure that the microbial communities multiply and are active. Nutrients such as potassium and magnesium are known to have an important physiological function in methanogens. Potassium is used to improve cell wall permeability (Kayhanian & Rich, 1995; Scherer et al., 1983). Magnesium is found in high concentration in methanogens and serves as a cofactor for certain enzymatic reactions (Scherer et al., 1983).

Examples of essential micronutrients include iron, nickel, cobalt, molybdenum, selenium and tungsten (Fermoso et al., 2009). Extracellular enzymes carry out the hydrolysis process and their activities are associated with the presence of these micronutrients, which are known to

form part of the enzyme active site (Wu et al., 2016a). Other functions include the ability to serve as agents binding nutrients such as phosphatases (Oleszkiewicz & Sharma, 1990). Although these trace elements are important, they are required in low concentration by microbes during AD. For example, during the AD of sewage sludge, the presence of nickel at the required concentration ensures good performance and process stability (Demirel & Scherer, 2008). However, at a concentration greater than 1 gm^{-3} , nickel inhibited methanogenesis (Demirel & Scherer, 2008).

2.6. General operating parameters of anaerobic digestion

As discussed in section 2.5, AD is a complex process that is carried out by various microbial communities that require different conditions (Yadvika et al., 2004). Therefore, maintaining specific microbial communities in a reactor is essential for optimal biogas production (Weiland, 2010). However, a number of factors that influence or affect microbial activity may limit the efficiency of AD (Leung & Wang, 2016). Numerous operational conditions are required for AD process stability (Leung & Wang, 2016). Due to the importance and benefits that the technology has to offer, many studies have been conducted in the interests of simplifying and optimising the process (Bolzonella et al., 2003; Chuang et al., 2011; Kameshwar & Qin, 2016; Lastella et al., 2002; Sahito et al., 2013; Usack et al., 2012). The operational conditions, such as temperature, pH, C: N ratio, organic loading rate and hydraulic retention time, were all found to be very important in maintaining process stability (Yadvika et al., 2004). It is thus essential that these operational parameters are maintained throughout the AD process for optimal biogas production. The operational parameters are discussed in sections 2.6.1 to 2.6.5.

2.6.1 Temperature

The literature cites different temperature ranges that have been used for the AD process. These include the psychrophilic ($10\text{--}20^{\circ}\text{C}$), mesophilic ($20\text{--}40^{\circ}\text{C}$) and thermophilic ranges ($50\text{--}60^{\circ}\text{C}$) (Guo et al., 2014; Sibiya et al., 2014). The mesophilic temperature with an optimum range of 30 to 35°C , and the thermophilic range, with an optimum range of $\geq 50^{\circ}\text{C}$, are commonly used for maximum biogas yield. These different optimal temperatures can be attributed to the uniqueness of the microbial communities, which operate optimally under specific temperatures, thus affecting biogas production (Chuang et al., 2011).

2.6.2 pH

Although the pH of the substrate in the digester reflects the state of the AD, it is not suitable as an early indicator of process instability (Brown & Li, 2013). Methanogenesis is carried out

by microbial communities that are sensitive to decreases in pH (Sibiya et al., 2014). A pH between 6.5 and 7.5 is considered to be optimal for the AD process (Wang et al., 2014). Although each of the stages is carried out by different microbial communities, all these microbial communities perform optimally at the above-mentioned pH (Leung & Wang, 2016). The first three stages, known as the acidification stages, result in the production of the organic acids, such as volatile fatty acids (VFAs), which are responsible for the decrease in pH (Brown & Li, 2013).

2.6.3 Carbon-nitrogen (C/N) ratio

During AD, microbial activities are maintained by the availability of nutrients such as carbon, nitrogen, phosphorus and potassium. Carbon and Nitrogen, however, are considered to be the most limiting nutrients. Therefore, the C/N ratio is an important indicator for controlling AD and is defined as the mass of carbon to the mass of nitrogen available in the feedstock to be used in the AD process. Carbon serves as a source of energy while nitrogen is used for microbial growth (Leung & Wang, 2016). Thus, low nitrogen content is associated with a slow increasing microbial community and high nitrogen content with a fast increasing microbial community (Leung & Wang, 2016). However, the breakdown of the substrate with high nitrogen content results in the production of elevated levels of ammonia, which must be avoided in the AD process as ammonia is known as an inhibitor of the AD processes (Yenigün & Demirel, 2013).

Improper C/N ratios in AD are usually associated with poor buffering capacity and the possibility of VFAs accumulation, resulting in a pH decrease and the eventual AD process failure (Wang et al., 2014). Improper C/N ratios are usually caused by the use of a single substrate for AD with high a nitrogen content (Leung & Wang, 2016). Therefore, to avoid the production of excess ammonia that might result in process instability, the buffering capacity may be improved by adding carbon-rich feedstock (Rincón et al., 2008). Improvement in buffering capacity can be obtained through co-digestion.

The use of different substrates in co-digestion is important since various substrates differ in their carbon and nitrogen content (Wang et al., 2014). Kumar (2005) showed that feed from a mixture of water hyacinth and night soil had improved the nitrogen, phosphorus and potassium in comparison to mono-digestion of water hyacinth. In addition, Patil et al. (2011) reported that water hyacinth is a good biogas producer but blending it with poultry waste significantly

increased biogas yield. Co-digestion and C/N ratio are, therefore, very important in process stability and optimum biogas production.

2.6.4 Organic loading rates

In continuous AD, digesters are fed continuously and organic loading rates (OLR) become important (Rincón et al., 2008). Organic loading rate is the quantity of organic matter fed into the digester per unit volume of the digester (Chen et al., 2014). Because microbial communities are the main drivers of AD, it is important to take note of the OLR due to community-specific organic degradation capacity (Chen et al., 2014). The lower the OLR the lower the biogas productivity while, in instances where the OLR is too high, organic overloading is experienced. Rincón et al. (2008) evaluated the effect of OLR and observed that, when OLR was increased, this resulted in process instability due to VFAs (up to 6.0 g/L) with the VFAs being assumed to be toxic to methanogens.

Organic overloading occurs when the concentration of organic matter added exceeds the degradation capacity of the microbes in the digester (Chen et al., 2014). Microbial degradation of organic matter in AD occurs in a series of steps although the growth rate of acid-forming bacteria is faster than that of methanogens (Chen et al., 2016). Organic overloading results in the increased population of organic acid bacteria, and the production as well as the accumulation of VFAs that causes the pH of the digester to decrease (Rincón et al., 2008). Organic overloading and the resultant acidification may be caused by changes in substrate ratio and composition as well as incorrectly measured substrate inputs or increased mixing rate which may lead to the inclusion of undigested material, such as floating layers into the digestion process.

2.6.5 Hydraulic retention time

Another important parameter is the average time that the feedstock remains in the digester – hydraulic retention time (HRT). The degradation capacity of the microorganisms depends on the retention time (Dereli et al., 2012). When the digestate (the remaining biomass after the anaerobic digestion of organic matter) is removed, active microorganisms are also removed (washed out). In a continuous process, if the HRT is not adequate, this may lead to hydraulic overloading (Dereli et al., 2012). This occurs when the HRT is insufficient for the multiplication of anaerobic microorganisms such as slow-growing methanogens. Insufficient HRT may eventually lead to the acidification of the digester through accumulation of VFAs. It is important for such process instability to be detected in time to avoid process failure. A

method such as FOS/TAC ratio is one of the methods used to monitor the stability of the digester (Rincón et al., 2008). The FOS/TAC parameters represent the ratio between volatile, organic, acids-equivalent acetic acid, and the buffering capacity – calcium carbonate (CaCO_3) (Scano et al., 2014). When the FOS/TAC ratio is between 0.3 and 0.4 (equiv. acetic acid/equiv. CaCO_3), the process is considered to be in good operating conditions without acidification risk (Rincón et al., 2008).

The average time a substrate spends in the digester for biodegradation and conversion to biogas depends on the temperature used and the type of substrate. Njogu et al. (2015) explained that temperature determines the HRT based on the type of substrate used. For example, psychrophilic digestion has an estimated HRT of over 100 days, mesophilic over 20 days, while thermophilic over 8 days (Njogu et al., 2015). Substrates may also affect the selection of HRT. In such instance, a biodegradable substrate with low total solids (TS) content may have a short HRT as compared to recalcitrant substrates.

2.6.6 Anaerobic digestion imbalances

Anaerobic digestion imbalances could arise when VFAs concentration exceeds the buffering capacity of the components in the digester. Characterisation of the feedstock for pH, TS, volatile solids (VS), VFAs, C/N ratio and water content is very important to provide relevant information about the content of the feedstock before use. Such analyses may also be measured throughout continuous AD. Total solids refers to the amount of suspended and dissolved solids that may affect the activity of anaerobic microorganisms. In wet fermentation method, which represents the majority of the existing biogas production processes, the TS content of the feed should not exceed 10% (w/v). Yi et al. (2014) showed that the substrate TS affects the performance of AD and that the change in TS content leads to a change in the microbial community structure in the AD system. Volatile fatty acids are intermediate metabolites that are produced during acidogenesis and are precursors of CH_4 . Their reduction in digestate implies that they were converted to CH_4 . The general operating parameters of AD provide an overview of process stability and allow for informed decisions to be made on process modification to prevent process failure.

2.6.7 Water hyacinth as feedstock for biogas production

It has been established by a number of researchers that water hyacinth can be converted into biogas (Almoustapha et al., 2009; Kumar et al., 2008; Kunatsa et al., 2013; Kurniawan et al., 2014; Lay et al., 2016; Njogu et al., 2015; Ofoefule et al., 2009; Patil et al., 2014; Singhal &

Rai, 2003; Wang & Calderon, 2012). Currently, emphasis has been placed on finding pre-treatment methods for enhancing hydrolysis as well as improving biogas yield with minimal cost (Ofoefule et al., 2009; Patil et al., 2011). Physical, chemical and biological pre-treatment methods have all been tested on water hyacinth with various studies observing different yields of biogas linked to the method used (Almoustapha et al., 2009; Ofoefule et al., 2009; Patil et al., 2012; Patil et al., 2011).

A study by Ofoefule et al. (2009) compared a combination of physical and chemical pre-treatment (A), physical and partial decomposition pre-treatment (B) and physical and partial decomposition combined with cow dung (C) in improving biogas production. Treatment C produced the highest biogas yield followed by treatment B. However, treatment A did not improve biogas yield when compared to the other treatments as well as the control. It was, therefore, concluded that water hyacinth does not require chemical pre-treatment. Patil et al. (2011) evaluated the effects of different pre-treatments (physical and chemical) on biogas yield and suggested that dried and chopped water hyacinth combined with poultry waste had the highest biogas yield as compared to both water hyacinth treated with sodium hydroxide and the untreated water hyacinth.

It is evident from all the studies discussed that water hyacinth is a potentially promising feedstock for biogas production. Water hyacinth requires minimal pre-treatment to improve biogas yield. This is very important because simple physical pre-treatment, such as chopping and milling, and biological pre-treatment, such as the use of naturally occurring aerobic microorganisms, may assist with reducing the pre-treatment cost and still result in high biogas production. A common AD optimisation method that was tested in the studies discussed was the co-digestion of the treated water hyacinth with other substrates (such as cow dung). Co-digestion serves as an advantage in biogas production because the use of single organic substrates may result in a number of drawbacks such as the improper carbon-nitrogen (C/N) ratio and poor buffering capacity that may affect biogas yield and, ultimately, lead to system failure (Wang et al., 2014; Wang et al., 2012).

Kumar (2005) showed that feed from co-digestion of water hyacinth and night soil had improved the nitrogen, phosphorus and potassium as compared to feed from water hyacinth alone. The ratio of water hyacinth to night soil that was used was 3:1 and it was suggested that the plant could be used as a major feed for AD with other organic substrates used to help with buffering capacity. Patil et al. (2011) reported that water hyacinth is a good biogas producer but that blending it with poultry waste significantly increased biogas yield while Wang et al.

(2014) reported that the use of a single substrate might affect the efficiency of AD due to an insufficient amount and the diversity of organic matter. Physical pre-treatment and co-digestion are methods that may be used to improve the yield and stability of the AD of water hyacinth with minimal cost. Pre-treatment increases the rate of hydrolysis, while co-digestion helps through the combination of nutrients required either to reach an optimal balance for AD or it may help to establish the required moisture content and organic diversity that may assist in controlling the pH (Alvira et al., 2010; Harmsen et al., 2010; Kennedy et al., 2015).

In conclusion, water hyacinth is an unwanted aquatic plant but using it as a feed for biogas production could reduce the challenges associated with the plant. Water hyacinth, like any other biomass, is renewable and, due to its elevated proliferation rates, has the potential to be a sustainable and environmentally friendly source of energy (Harun et al., 2011). Challenges of low biogas conversion efficiency make it necessary for the continuous optimisation of the AD process to improve biogas production when water hyacinth is used as feedstock.

CHAPTER 3

METHODOLOGY

3.1. Permit application and ethics approval

Water hyacinth is an invasive aquatic plant which is not indigenous to South Africa. As part of the requirement for the University ethics application, a permit was required which allowed for the collection and transportation of water hyacinth from Hartbeespoort Dam, North West Province, to a microbiology lab in Pretoria, Gauteng, South Africa. Two permit documents were issued by the Department of Environmental Affairs, South Africa.

Permit 1: General permit conditions for research purposes involving alien and invasive species- Permit number: 5086577918 (Appendix A3.1).

Permit 2: Conditions for conveying, moving or otherwise translocating water hyacinth- Permit number: 5086577921 (Appendix A3.2).

The two permits, together with the required ethical application form, were used to apply for ethics approval to carry-out the study. This was granted by the University of South Africa Research Ethics Review Committee (Appendix A3.3).

3.2. Study area

The Hartbeespoort Dam, located in the North West province of South Africa, was chosen as the study site (Figure 3.1). The dam is utilised primarily for domestic, industrial, agricultural and recreational purposes (DWA, 2012) and contributes to the economy of North West. However, at the time of the study, the dam was in a hypertrophic state (rich in nutrients) caused by the surrounding environments (van Ginkel, 2011) leading to a significant invasion by water hyacinth. Since the complete removal of the plant is almost impossible due to the ability of the plant to produce seeds with the ability to remain viable for up to two decades (Bhattacharya et al., 2015), the plant has the potential to be used as continuous feed for renewable energy production.

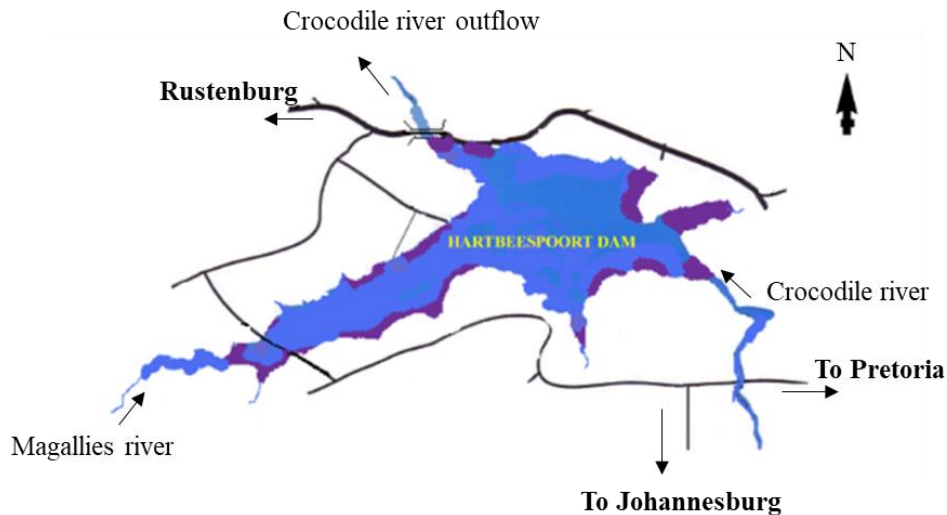


Figure 3.1: Hartbeespoort Dam located in the North West Province of South Africa

3.2.1 Sample collection

3.2.1.1. Water hyacinth

Fresh water hyacinth (whole plant) was harvested from the Hartbeespoort Dam (25°44'51"S 27°52'1"E) (Figure 3.2). As a precaution to prevent water hyacinth spread to other water bodies, the plants were harvested and transported in sealed containers. On arrival at the microbiology laboratory at the Agricultural Research Council (ARC)-Institute for Soil, Water and Climate (ISCW), tap water was used to manually wash the plant to remove unwanted particles, and the used water and the remaining plant materials were autoclaved before being discarding.

3.2.1.2. Cow dung

Cow dung was collected aseptically by hand at ARC- Animal Production Institute (API) and placed in a cooler box. The collected samples were transported to the ARC-ISCW microbiology laboratory for storage at 4°C until used.



Figure 3.2: Water hyacinth harvesting (Hartbeespoort Dam April 2017)

3.3. Chemical analysis of untreated water hyacinth

The cleaned plant samples were separated into three portions, namely, the leaves, petioles and roots, to evaluate compositional differences of the various plant parts. The three portions of the samples were oven dried separately for 24 hours at 105°C. The samples were then ground and analysed for macronutrients (nitrogen, carbon, phosphorus and potassium). The plant samples were also subjected to a micronutrient analysis scan for the determination of the available micronutrients. These analyses were carried out at ARC-ISCW analytical services using inductively coupled plasma mass spectrophotometry (ICP-MS), where 1 g of the samples was digested using 21 ml of Nitric acid (HNO₃). The whole plant was also evaluated for its chemical composition (proteins, fats, carbohydrate, lignin, hemicellulose and cellulose) at the ARC- API analytical services using the Association of Analytical Communities (AOAC) official methods 920.39, 934.01, 930.15, 942.05 and 954.01 (Greenfield & Southgate, 2003; Robertson, 1981; Harris, 1970). Three procedures were used, namely, the neutral detergent fibre (NDF), the acid detergent fibre (ADF) and the acid detergent lignin. The NDF measures most of the structural components of the plant cell, that is, the lignin, cellulose and hemicellulose and the method is based on the extraction of feed with a hot neutral solution of sodium lauryl sulphate. The ADF mainly measures the cellulose and lignin content and the method is based on heat-treating the sample with sulphuric acid containing cetyltrimethylammonium bromide. Whereas the ADL measured the lignin, content and is determined by oxidation with potassium permanganate. The difference between NDF and ADF

values give an estimation of hemicellulose content whereas the difference between ADF and ADL values gives an estimation of cellulose content.

3.4. Effects of pre-treatment on water hyacinth composition

The cleaned harvested water hyacinth plants were subjected to four physical as well as a combination of physical and biological pre-treatment methods (Table 3.1). The physical pre-treatment focused primarily on varying the particle size, while a combination of physical and biological pre-treatment focused on size reduction as well as the use of naturally occurring aerobic microorganisms to release the sugars. Each pre-treated sample was tested for chemical composition (dry matter, carbohydrates, protein, fats, cellulose, hemicelluloses, lignin, and ash) and the results were compared to evaluate the impact of pre-treatment on the chemical composition. The chemical composition was analysed as described in section 3.2.

Table 3.1: Summary of the five pre-treatments

Samples	Pre-treatment	Condition
Physical pre-treatment		
H	Homogenised using a pestle and mortar	Wet
HC	Chopped using a pair of scissors	Wet
OD	Oven-dried at 105°C for 24 hours and powdered	Dry
SD	Sun dried for 7 days and chopped	Partially dry
Combination of physical and biological pre-treatment		
HCD	Chopped and allowed to decompose aerobically for 7 days	Wet

3.5. Batch assay

3.5.1 Inoculum to substrate ratio (ISR)

The inoculum used in this study was collected from a running 20 L mesophilic lab-scale anaerobic semi-continuous stirred tank reactor (CSTR) with a working volume of 14 L. The reactor was fed every alternate day with 140 g fresh cow dung and water at a ratio of 1:1. The performance of the digester in which the inoculum was collected was stable with an average methane yield of 1450 L gVS⁻¹.

3.5.2 Batch anaerobic digestion

Standard curves were constructed to ensure that each treatment contained 2% TS (due to the different moisture content, each gram of the five pre-treatments differed in the amount TS content). Two percent TS of the oven-dried pre-treatment method was used as the reference dry weight of water hyacinth. The amount of substrate to be weighed for the other pre-treatment methods containing 2% TS were calculated using the equations as depicted on the graphs in Appendix A3.4.

A series of batch anaerobic digestion trials were conducted using lab-scale 500 ml glass anaerobic digesters with 250 ml working volume. The batch digesters were set up as follows: 1 - pre-treated water hyacinth and inoculum, 2 - pre-treated water hyacinth without inoculum and 3 - inoculum only (Table 3.2). Anaerobic digestion of the water hyacinth with actively digested cow dung slurry (inoculum) was conducted at a ratio of 3:1 (water hyacinth: inoculum) with total solids of 2%. The pre-treated substrate (HC, H, OD, SD and HCD) with the addition of inoculum is henceforth be referred to as ‘treatments’ and the pre-treated substrate without inoculum is referred to as controls (HC control, H control, OD control, SD control and HCD control). All assays contained total solids (TS) of 2% and were conducted in triplicate. The mesophilic temperature range was chosen because less energy input is required for heating than thermophilic AD (Levén et al., 2007). Tap water was used to make the volume up to 250 ml. Anaerobic conditions inside batch bottles were created by purging the bottles with nitrogen for 3 minutes. Thereafter, the bottles were sealed with a lid equipped with rubber septa (Silicone cream/PTFE beige, Hardness 55°, shore A, Thickness 3.2mm) (Monitoring & Control Laboratories (PTY) LTD). The digesters were continuously mixed at 130 rpm for substrate and heat distribution during an incubation period of 35 days.

3.5.3 Evaluation of biomethane yield

Gas chromatography (SRI 8610C) was used to analyse the composition of the biogas. The instrument is equipped with a HayeSep D packed column and thermal conductivity detector. The method adopted involved different stages. The oven had an initial temperature of 50°C held for 4 minutes, initial ramp temperature at 20°C per minute and final temperature at 220°C. The thermal conductivity detector was operated at 155°C with a reference flow of 20 ml per minute and a make-up flow of helium gas at 10 ml per minute. Biogas samples were taken using a 5 ml gas tight syringe with Luer lock valve (SGE 10MDR-VLLMA-GT) to obtain the

percentage of CH₄ and CO₂ produced. At the beginning and end of digestion trials, the pH of the substrate and digestate were measured using a pH meter (AD1030).

3.5.4 Statistical analysis

The data was subjected to an appropriate analysis of variance (ANOVA). The Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro & Wilk, 1965). Student's t-LSDs (Least significant differences) were calculated at a 5% significance level to compare the means of the significant source effects (Snedecor & Cochran, 1967). The above analysis was performed using Genstat Release 18 and SAS version 9.3 statistical software (SAS, 1999).

3.6. Semi-continuous anaerobic digestion

The batch section showed that the treatments and the controls were able to produce CH₄, although the CH₄ production of the controls was slow at the beginning of the study due to the absence of inoculum. In semi-continuous AD, the ability of the controls to produce CH₄ without process failure was the motivation for evaluating the mono-digestion of the plant in comparison to the co-digestion. In addition, to avoid long lag phase as observed in the controls (the cause of slow CH₄ production at the beginning of the process), the inoculum was added.

3.6.1 Inoculum preparation

The inoculum was prepared using a batch reactor operating at a working volume of 3 L. The reactor was initially fed with 10% of fresh cow dung and water (w/v) at a ratio of 1:1. The reactor was incubated at a mesophilic temperature for a period of 3 weeks while continuously mixed at 130 rpm for substrate and heat distribution. The performance of the digester was stable with a cumulative CH₄ production of 0.24 L.

3.6.2 Evaluation of the effect of organic loading rates on semi-continuous AD

The effect of varied organic loading rates (OLR) on microbial community structure and composition, process performance (biogas production) and stability (FOS/TAC ratio and pH) were evaluated. Two 20 L anaerobic semi-continuous stirred tank reactors (CSTR) operating at a working volume of 10 L were used. The two digesters had identical dimensions and configurations (Figure 3.3). Digester 1 contained hand cut water hyacinth (mono-digestion) while digester 2 contained hand cut water hyacinth and fresh cow dung (co-digestion) mixed at a ratio of 3:1 respectively (Kumar, 2005). A volume of 200 ml of inoculum was used for activation in both digesters and included a start-up period of 17 days (to allow microbial

community to increase and produce CH₄). Once microbial community and their function had been established, (based on the biogas and CH₄ production) semi-continuous feeding was initiated (collection of 10% digestate and refill with 10% substrate). Semi-continuous feeding with an OLR of 1.24 and 1.47 gVS⁻¹ for mono- and co-digestion respectively once a week was conducted for a period of 21 days (stage 1). Once microbial communities had adapted to the environment, disturbances were initiated where the OLR was increased to twice a week (stages 2). The microbial communities were also allowed to adapt for another 21 days and the OLR was changed again (stage 3). During stage 3 the OLR was reduced to once a week, however, the VS were increased to 2.34 and 2.98 gVS⁻¹ for mono- and co-digestion respectively. The type of AD was wet fermentation with stages 1 and 2 containing approximately 2% total solids (TS) and stage 3 approximately 4% TS. The calculations of the VS were based on the percentage of TS used (2% and 4%). The VS in co-digestion were higher and this may be explained by the fact that TS does not contain the same amount of VS (Frigon & Guiot, 2010). Biogas production was measured using the gas counter connected to the digesters for digester performance. The biogas content was analysed as explained in the section on batch assay. The total biogas production was measured using the gas counter connected to the digesters while the CH₄ production in litres was calculated using the biogas produced after determining the content of the headspace gas (%) (Duran et al., 2006). The CH₄ yield was calculated to measure the volume of CH₄ produced relative to the amount of volatile solids added. The CH₄ yield was calculated using equation 1 below (Nurliyana et al., 2015).

$$\text{Methane yield (L CH}_4\text{/gVS)} = \frac{\text{Cumulative volume of CH}_4 \text{ (LCH}_4\text{)}}{\text{Mass of original VS added (gVS)}} \dots\dots\dots(1)$$

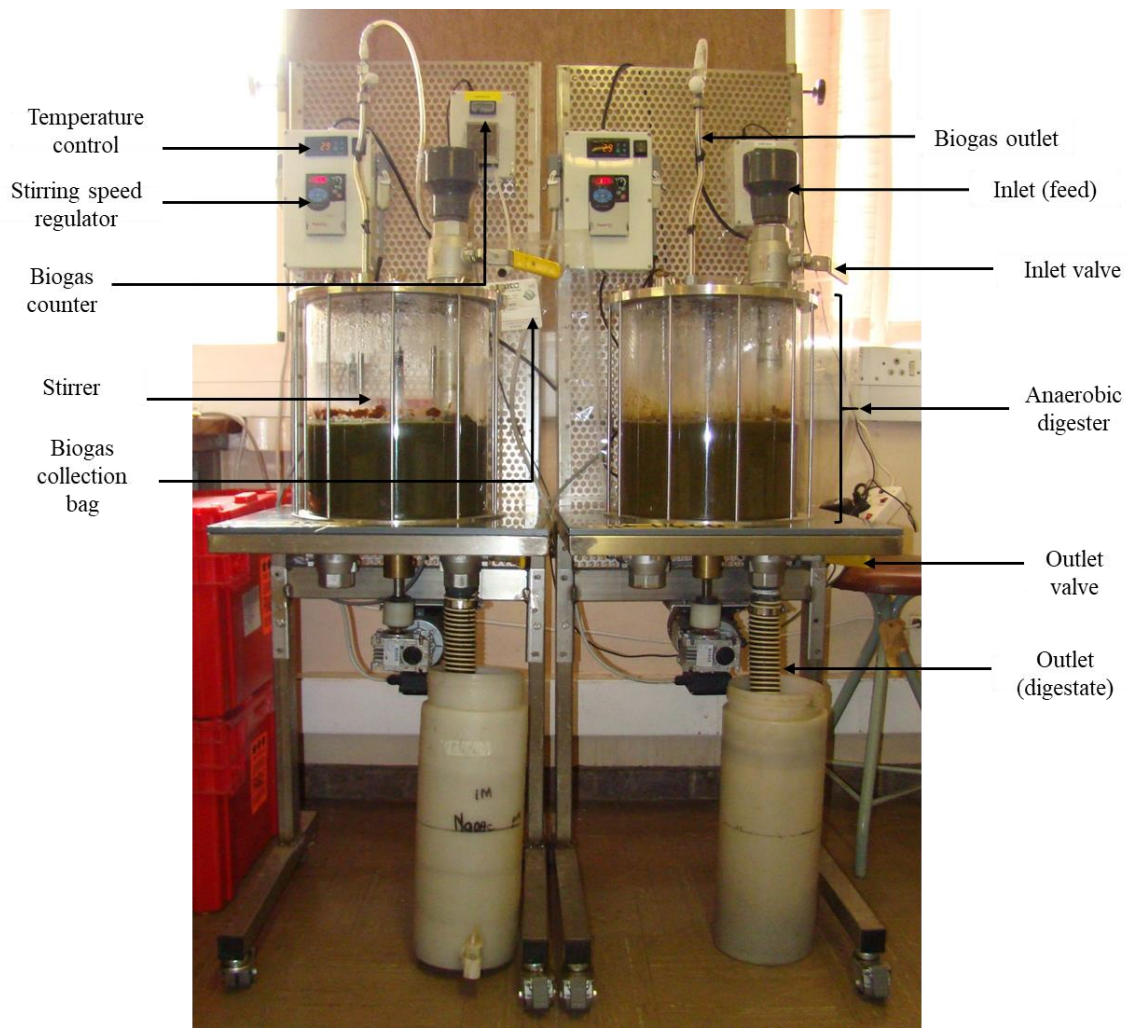


Figure 3.3: Semi-continuous anaerobic digesters with identical dimensions and configuration

3.6.3 Assessment of process stability using the FOS/TAC ratio and pH

A potentiometer titrator was used to calculate the FOS/TAC ratio using formula 2 and 3. These formulae (2 and 3) were pre-programmed in the 877 Titrino plus titrator (Metrohm, USA) (Lossie & Pütz 2008). The FOS/TAC ratio was calculated every alternate day. The digestate volume of 30 ml was sampled out of the reactors and centrifuged at 2700 rpm for 20 minutes to remove any coarse components. Five millilitres of the supernatant was diluted in 35 ml of distilled water in a beaker. The beaker containing the diluted supernatant was continuously homogenised during the titration process. The titration was conducted by the addition of 0.1 N H_2SO_4 until a pH of 5 was reached and the volume of acid added was noted. Thereafter, the titration continued until a pH of 4.4 was reached and the volume of acid added was again noted. The pre-programmed formulae were used to automatically calculate the FOS/TAC ratio which is commonly used for monitoring AD process stability (Allen et al., 2014; Lossie & Pütz 2008; Rincón et al., 2008; Scano et al., 2014; Voß & Weichgrebe, 2009). The FOS/TAC parameters

represent the ratio between volatile organic acids-equivalent acetic acid, and the buffering capacity- calcium carbonate (CaCO_3) (Scano et al., 2014). A ratio of between 0.3 and 0.6 (equiv. acetic acid/equiv. CaCO_3) for renewable raw material is related to maximum biogas production and implies process stability. Whereas a FOS/TAC ratio below 0.3 may be related to low biogas production which implies process instability (Lossie & Pütz 2008; Rincón et al., 2008). The 877 Titrino plus titrator (Metrohm, USA) was also used to measure the pH sample before titration.

$$\text{TAC} = H_2SO_4 - \text{volume added from start to pH 5 in ml} \times 250 \dots\dots\dots(2)$$

$$\text{FOS} = (H_2SO_4 - \text{volume added from pH 5 to pH 4.4 in ml} \times 1.66 - 0.15) \times 500 \dots\dots\dots(3)$$

3.7. Microbial community analysis

The digestate samples were collected in both digesters as depicted in Table 3.2 and stored at -20 until used. In stages 1 and 3, a retention time of 7 days was allowed before the next feeding, whereas in stage 2, a 4 days retention time was allowed

Table 3.2: Collection of digestate for microbial analysis

Sampling time	Stage 1	Stage 2	Stage 3
Early	3 days after initial feeding (day 20)	3 days after initial feeding (day 41)	3 days after initial feeding (day 62)
Mid	5 days after second feeding (day 29)	2 days after forth feeding (day 50)	5 days after second feeding (day 71)
Late	7 days after third feeding (day 38)	4 days after sixth feeding (day 59)	7 days after third feeding (day 80)

3.7.1 DNA extraction and gene amplification

Two millilitres of the collected digestate was centrifuged at 13000 rpm for 2 minutes. The supernatant was discarded and the pellet was used for genomic DNA (gDNA) extraction. gDNA was extracted from the samples collected during the continuous AD using DNeasy PowerSoil kit (Whitehead Scientific (Pty) Ltd), in accordance with the manufacturer's instructions. The extracted gDNA was quantified using Qubit 2.0 fluorometer (ThermoFisher, Edenvale, South Africa) (Appendix A3.5). The choice of DNA extraction kit for digestate was

based on a previous study by Roopnarain et al. (2017). The extracted DNA was stored at -20°C for further investigation.

The primers in Table 3.2 were used to amplify the following metabolic genes: Formyltetrahydrofolate synthetase (FTHFS), Methylcoenzyme M reductase (*mcrA*) and Acetyl-coA synthetase (ACAS). The metabolic gene sequences were amplified in a 25 µl reaction containing 5 µM of each primer, 12.5 µl of one *Taq* 2X master mix with standard buffer (Biolabs) and sterile distilled water to make up the 25 µl volume. PCR amplifications were performed at 94°C for 30 seconds, 30 cycles of denaturation at 94°C for 30 seconds, annealing at specific temperature given in Table 3.3 for 30 seconds and extension at 68°C for 1 minute; and final extension at 68°C for 5 minutes. Gel electrophoresis was used to view the amplified genes. Selected samples of the amplified metabolic genes were sequenced (to confirm identity) at Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa, using the ABI Big dye V3.1 kit according to the manufacturer's instructions and the ABI 3500XL genetic analyser using sequencing. The received sequences were compared to sequences of the GeneBank database for identity. The sequences were further aligned using ClustalW multiple alignment on BioEdit and MAFFT - a multiple sequence alignment programme, online version. The phylogenetic tree was constructed by the Neighbor Joining method with 1000 bootstraps.

Table 3.3: Primers used to amplify metabolic genes.

Primer	Sequence	Target metabolic gene	Target microorganisms	Annealing temperature (°C)
FTHFS_f	5'-TTYACWGGHGAYTTCCATGC-3'	FTHFS	Authentic Acetogens	53
FTHFS_r	5'-GTATTGDGTYTTRGCCATACA-3'			
mcrA_f	5'-TGTAACGACGGCCAGTGGTGGTGTGGMGGATTCA	mcrA	Methanogenic Archaea	51
mcrA_r	CACARTAYGCWACAGC-3' 5'-CAGGAAACAGCTATGACCTTCATTGCRTAGTTW GGRTAGTT-3'			
MSaeta_Aco-A_f	5'-TAATCCGCCAAAAGAGTTGG-3'	ACAS	Acetoclastic methanogen	56
MSaeta_Aco-A_r	5'-TCTTCTGGACTGGCTGGTCT-3'			

3.7.2 High-throughput sequencing of the 16S rRNA-gene

The primers 341F (5'-CCTACGGAGGCAGCAG-3') and 805(5'-GACTATHVGGGTATCTAATCC-3') with Illumina overhangs attached to the 5' end of the forward and reverse were used to amplify the hypervariable V3-V4 region of the 16S rRNA gene from the extracted DNA (Ezeokoli et al., 2018). The 16S rRNA gene was amplified in a 25 µl reaction containing 12.5 ng DNA template, 5 µM of each primer and 12.5 µl of Tempase HS 2X Master mix (Ampliqon, Denmark). PCR amplification was performed at 95°C for 3 minutes, 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. One percent agarose gel electrophoresis was used to view the amplified gene (Appendix A3.6). The prepared samples were submitted to the ARC-Biotechnology platform (Pretoria, South Africa), for subsequent processing and sequencing on the Illumina Miseq sequencer (Illumina Inc, CA, USA) using standard protocols.

3.7.2.1. Operational taxonomic units (OTUs) clustering and diversity analyses

The NGS sequences were inspected for quality using the FastQC software (v 0.11.7, Babraham Bioinformatics, UK). The forward and reverse reads were merged using PANDAseq (Masella et al., 2012). The merged reads were clustered into operational taxonomic units (OTUs) using “pick_open.reference_otus.py” script in QIIME while aligning against the SILVA rRNA (release 128) database (Quast et al., 2013) by using usearch61 and PyNAST aligner (Caporaso et al., 2010). The OTU table generated was exported to the R-studio for further statistical analyses. The R packages vegan, ape, labdsv and ggplot were installed and used for statistical analysis as well as plotting (Mashiane et al., 2017).

CHAPTER 4

RESULTS

4.1. Chemical analysis

4.1.1 Compositional analysis of water hyacinth

Four macronutrients were analysed in triplicates with the results showing varying compositions. The results showed that water hyacinth had a high carbon content in all the various plant parts with the leaves containing the highest carbon content at 38%. The nitrogen, phosphorus and potassium were below 5% in all 3 plant parts (Figure 4.1). The C/N ratio for the different parts of the plants was as follows; leaves – 9.8, petioles – 8.8 and roots – 12.3.

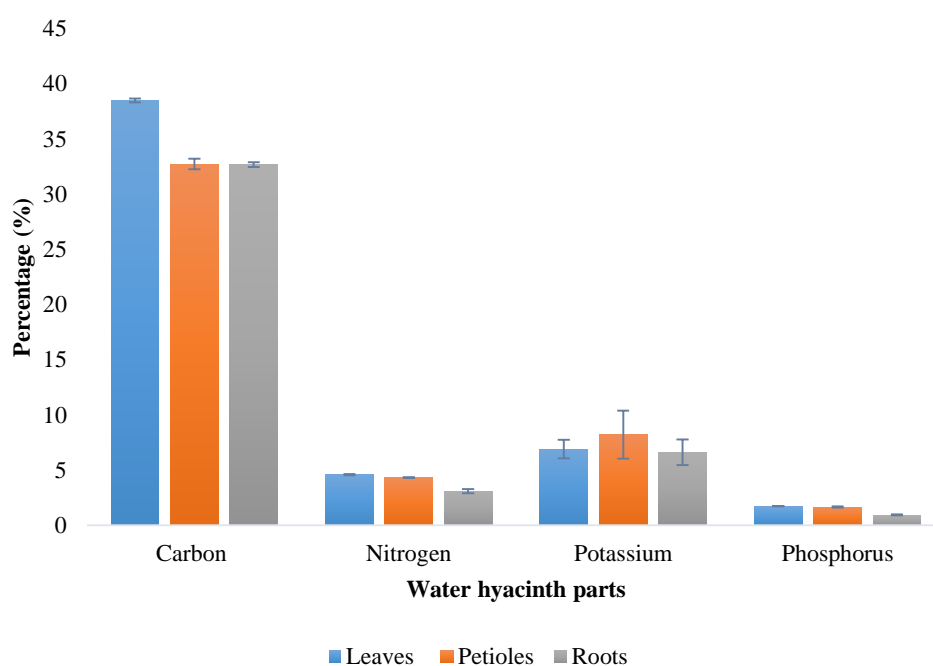


Figure 4.1: Chemical analysis of different part of the plant, error bars represent the standard deviation (n = 3)

4.1.2 Heavy metal scan

The mean concentration of heavy metals from the roots, petioles and leaves is represented in Figure 4.2 A and B. The roots contained more heavy metals as compared to the petioles and leaves. The plant showed high concentrations of manganese 4486.5, 372.4, 711.03 mg/kg for roots, petioles and leaves respectively (not represented in Figure 4.2 A and B). Other metals that were found in high concentrations included nickel, zinc, barium and titanium (Figure 4.2 B).

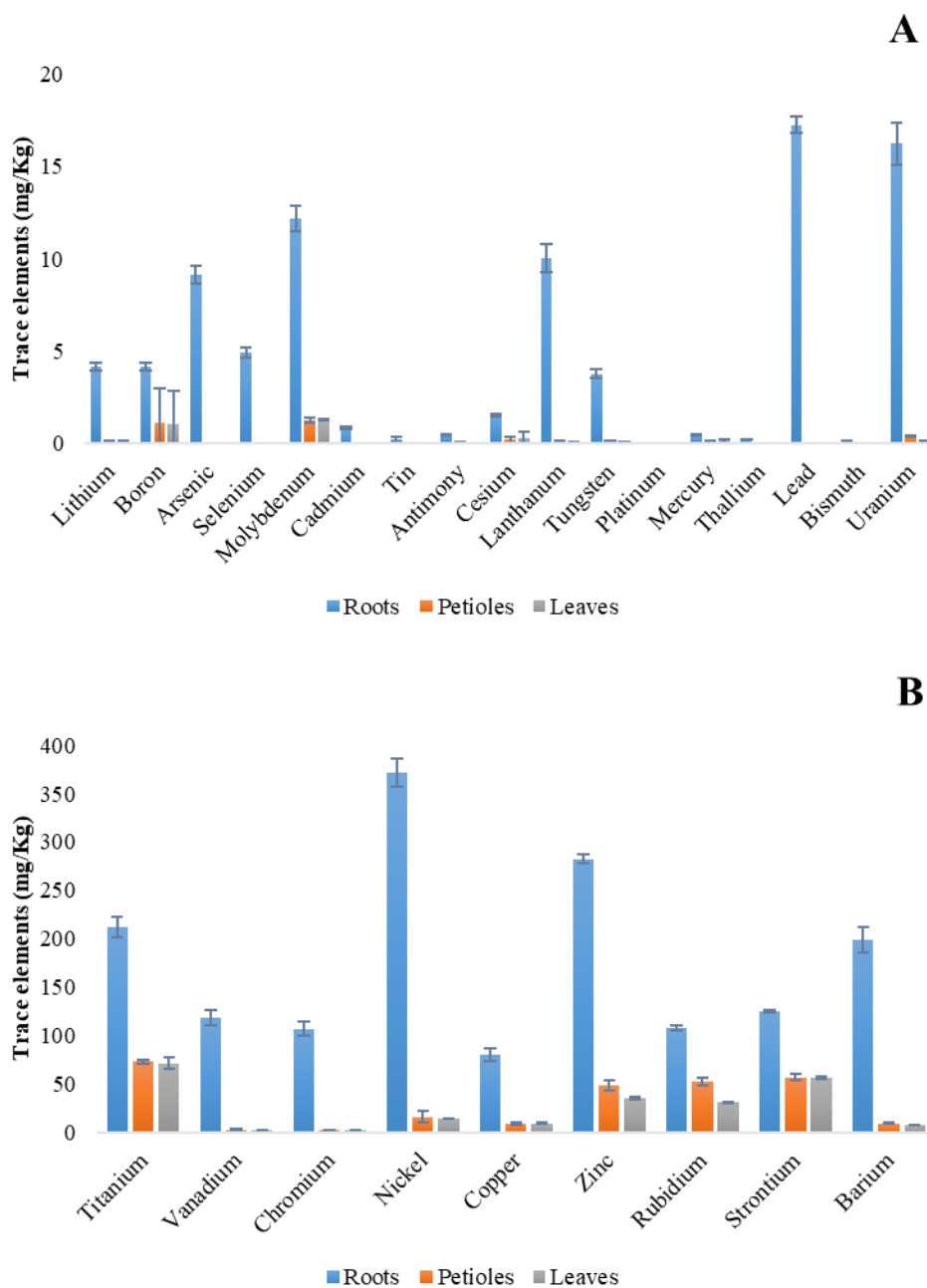


Figure 4.2: Trace elements from different parts of the plant. A – range of between 0.01–18 mg/kg and B – range of between 20–400 mg/kg. Error bars represent the standard deviation (n = 3)

4.1.3 Impact of pre-treatment on water hyacinth composition

The comparison of the components recovered after the pre-treatment methods is presented in Table 4.1. All the physical pre-treatment methods recovered approximately similar percentages of cellulose and hemicellulose (with the exception of OD for cellulose) whereas the HCD pre-

treatment method resulted in a lower recovery of cellulose. The lignin recovery was highest from HCD while SD resulted in the lowest recovery.

Table 4.1: lignocellulosic components of water hyacinth.

Components (% dry matter)	Physical pre-treatment				A combination of physical and biological pre-treatment
	H	HC	OD	SD	HCD
Cellulose	20.22	20.36	17.01	21.39	17.10
Hemicellulose	22.47	22.17	28.08	19.97	19.97
Lignin	4.87	7.69	9.57	3.87	10.88

4.2. Batch anaerobic digestion

4.2.1 Potential hydrogen (pH)

The pH of the treatments before and after AD is presented in Table 4.2. The mean pH was in a range of 7.2 to 7.6, with HCD having the highest pH value before AD. After AD, the pH of the treatments increased slightly to a range of 7.6 to 7.7, except for HCD in which the pH remained the same. A different trend was observed with the controls where the comparison of the pH after AD, in a range of 6.2 to 7.6, to the initial pH, in the range of 7.0 to 8.2, varied. For some controls, the pH increased while, for others, the pH decreased. The pH drop was the most pronounced in the H control where the pH decreased from 7.3 to 6.2.

Table 4.2: pH before and anaerobic digestion for the 5 pre-treatments

Pre-treatments	Before AD		After AD	
	Samples	Control	Samples	Control
HC	7.4 ± 0	7.7 ± 0	7.6 ± 0	7.5 ± 0.06
H	7.2 ± 0.06	7.3 ± 0.1	7.6 ± 0.1	6.2 ± 0.9
OD	7.2 ± 0.06	7.0 ± 0.06	7.7 ± 0.1	7.5 ± 0.06
SD	7.2 ± 0.06	7.0 ± 0.1	7.6 ± 0.06	7.4 ± 0.11
HCD	7.6 ± 0.23	8.2 ± 0.07	7.6 ± 0.06	7.6 ± 0
Inoculum	7.6 ± 0.06		7.2 ± 0.15	

*n = 3; \pm standard deviation

4.2.2 Evaluation of inoculum effect.

The cumulative CH₄ for the physical pre-treatment method assays is presented in Figure 4.3. The CH₄ production for the treatment H, HC, OD and SD started on day 3 and greatly increased until day 17 before stabilising. Treatments H, HC and OD produced the highest cumulative CH₄ of above 0.7 L while SD produced approximately 0.63 L on day 17. On day 35, SD produced the highest cumulative CH₄ of 0.95 L, while the cumulative CH₄ remained below 0.9 L for the remaining three pre-treatment methods. A different trend was observed in the controls, which slowly produced small amounts of CH₄ from day 3 to day 13. This increased slightly until day 21 before stabilising. However, the cumulative CH₄ production from the H control increased only slightly around day 29. From the controls, OD produced the highest cumulative methane of 0.4 L on day 21. However, on day 35, the highest cumulative CH₄ of 0.5 L was observed from the SD control whereas the H control produced the lowest amount of 0.19 L CH₄.

A similar trend was observed with the cumulative CO₂ (Figure 4.4) production for all the physical pre-treatments, although CO₂ production increased slowly to reach a maximum on day 13 before stabilising slowly. The highest CO₂ produced was 0.23 L - this was produced by SD on day 13. The controls produced more CO₂ than the treatment HC, OD and SD except for H in which the highest for the control and the treatment was the same. The inoculum produced lower amounts of CO₂ as compared to the treatments but, when compared to the CO₂ produced by the control, the inoculum CO₂ was higher. The inoculum produced a lower

amount of CH₄ and CO₂ as compared to the treatments but, when compared to the control, the amount of CH₄ and CO₂ produced by the inoculum was higher.

Cumulative CH₄ production for the combination of physical and biological pre-treatment methods (HCD) is presented in Figure 4.5 A. The cumulative CH₄ production started slowly on day 3 and increased until day 35. A cumulative CH₄ production of 0.35 L on day 17 was obtained and increased slightly to 0.49 L on day 35. The HCD control produced 0.25 L of CH₄, showing a similar trend observed in the H control from the physical pre-treatment methods. The cumulative CO₂ (Figure 4.5 B) production by treatment HCD was below 0.1 L for the whole incubation period while the HCD control CO₂ production slowly increased until day 29 before stabilising. The amount of CH₄ and CO₂ produced by the HCD and HCD controls were lower than the CH₄ and CO₂ produced by the inoculum.

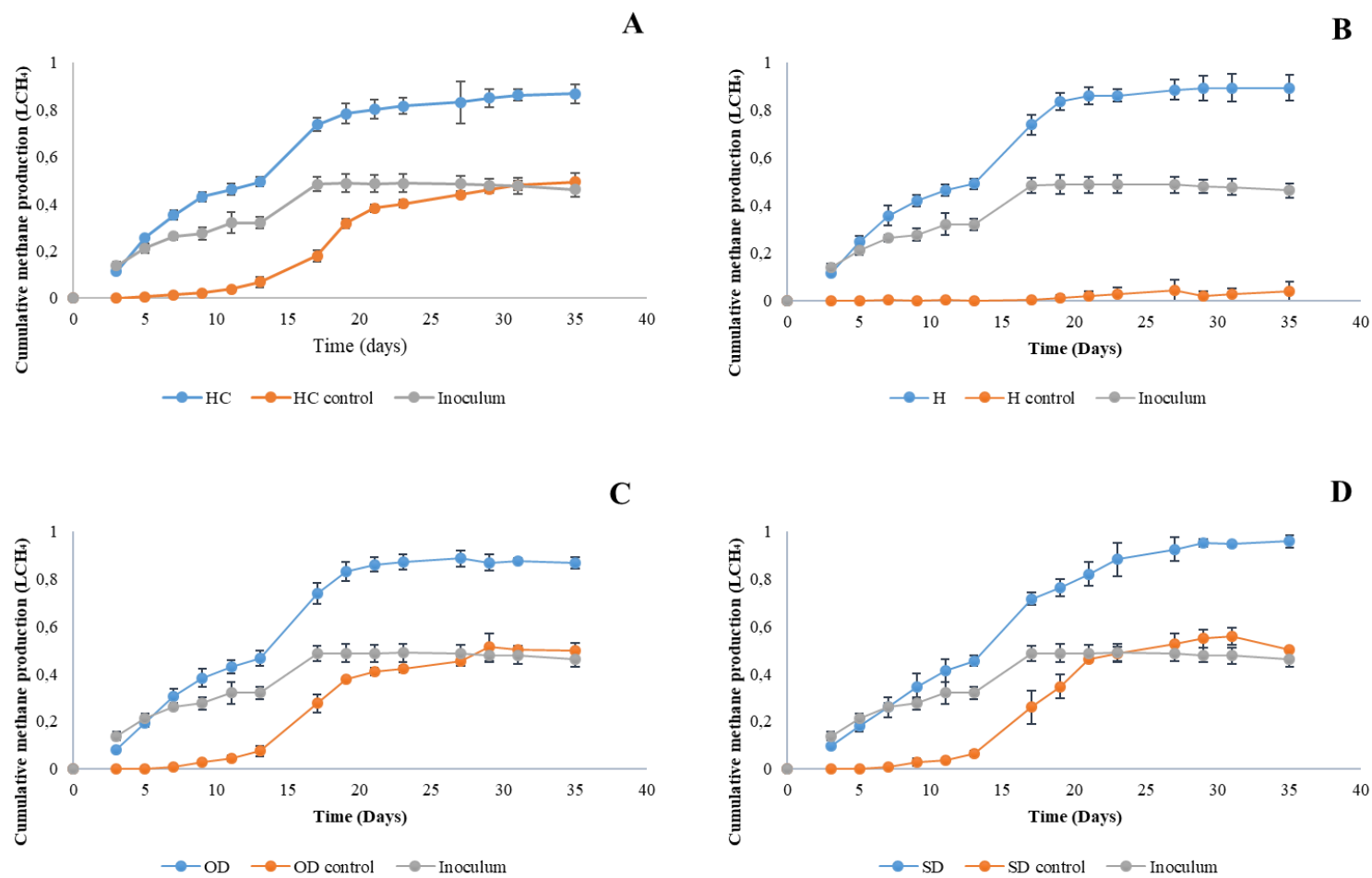


Figure 4.3: Cumulative CH₄ production for physical pre-treatment methods; A – hand cut, B – homogenised, C – oven dried and D – sun dried. Error bars represents the standard deviation (n = 3)

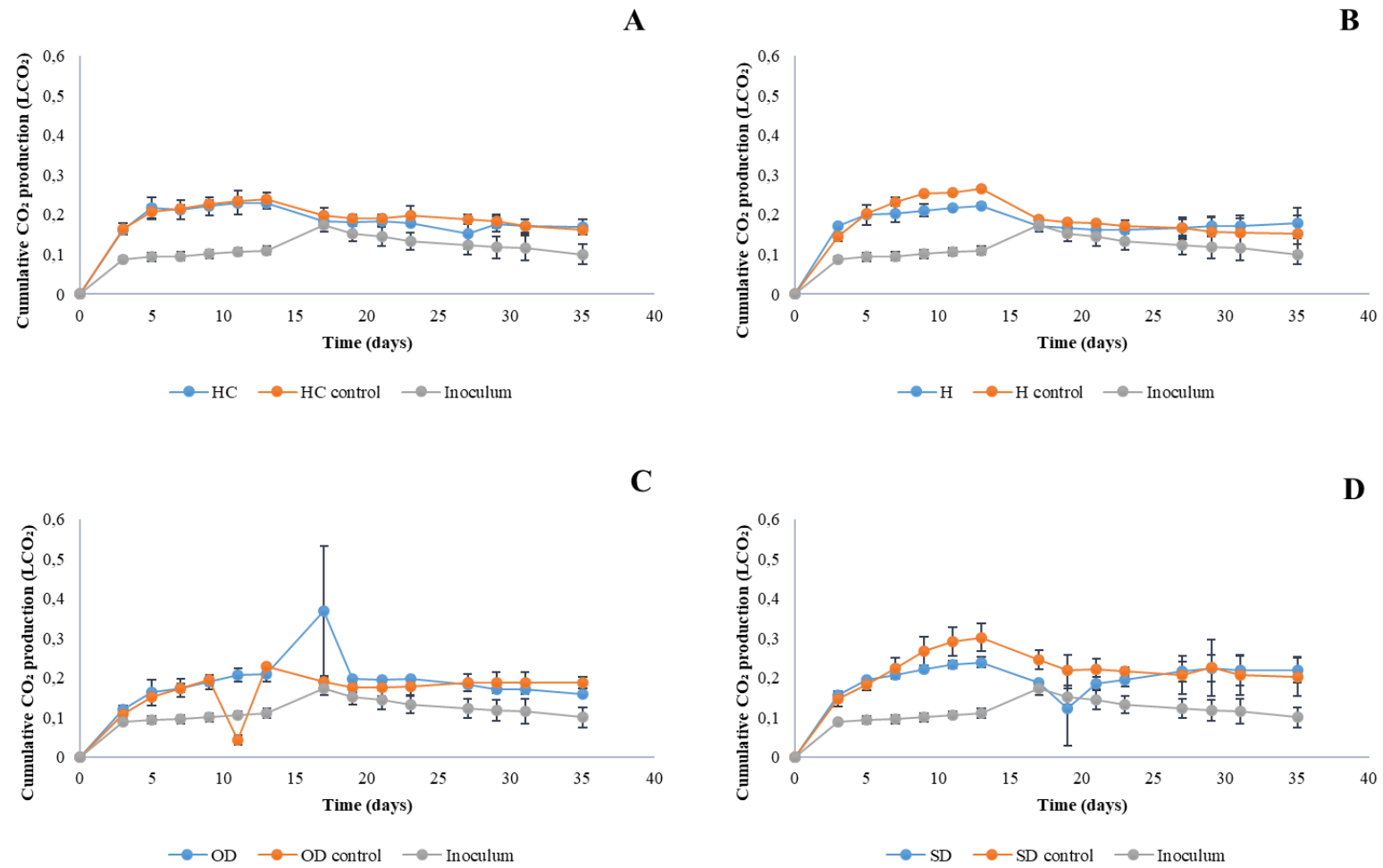


Figure 4.4: Cumulative CO₂ production from physical pre-treatment methods: A – hand cut, B – homogenised, C – oven dried and D – sun dried. Error bars represents the standard deviation (n = 3)

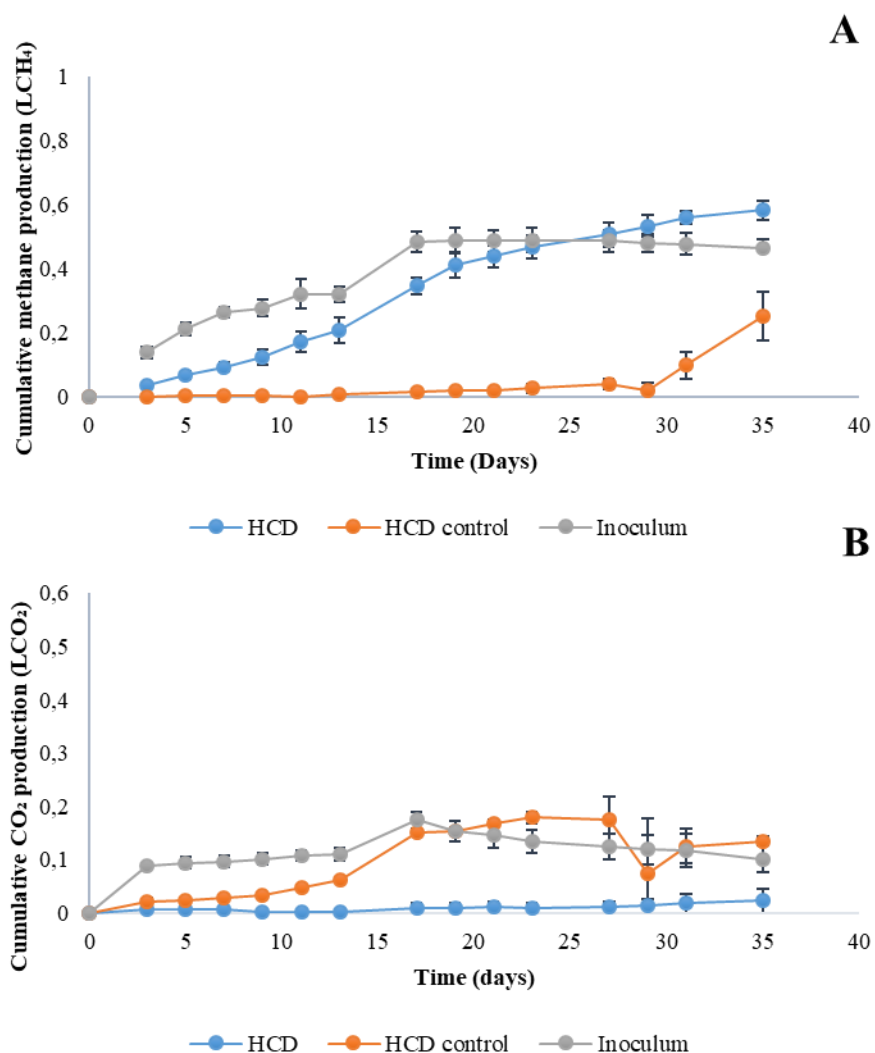


Figure 4.5: Cumulative CH₄ and CO₂ production from a combination of physical and biological pre-treatment methods (HCD): A – cumulative CH₄ and B – cumulative CO₂ production. Error bars represents the standard deviation (n = 3)

4.2.3 The effect of pre-treatment methods on biodegradability rates.

The five pre-treatment methods were compared for substrate biodegradability rates as well as their ability to enhance CH₄ production. The actively digested cow dung slurry, which served as the inoculum, was used as the control. The comparison, as indicated in Figure 4.6, showed that all the physical pre-treatment methods enhanced CH₄ production as compared to the combination of the physical and biological pre-treatment methods, which produced the lowest amount of cumulative

CH₄. Figure 4.6 also showed that the highest substrate biodegradability rates were observed for samples H, HC and OD. On day 17 there was no significant difference between all the physical pre-treatments, as well as no significant difference on day 35 ($P > 0.05$) for both CH₄ and CO₂ (Figures 4.7 and 4.8). However, a significant difference ($P < 0.05$) was observed when comparing day 17 and day 35 for cumulative CH₄ only. The cumulative CH₄ produced by HCD differed significantly ($P < 0.05$) for all the physical treatments on days 17 and 35 (Figures 4.7 and 4.8). However, when comparing the CH₄ and CO₂ produced by the HCD on days 17 and 35, a significant difference was observed.

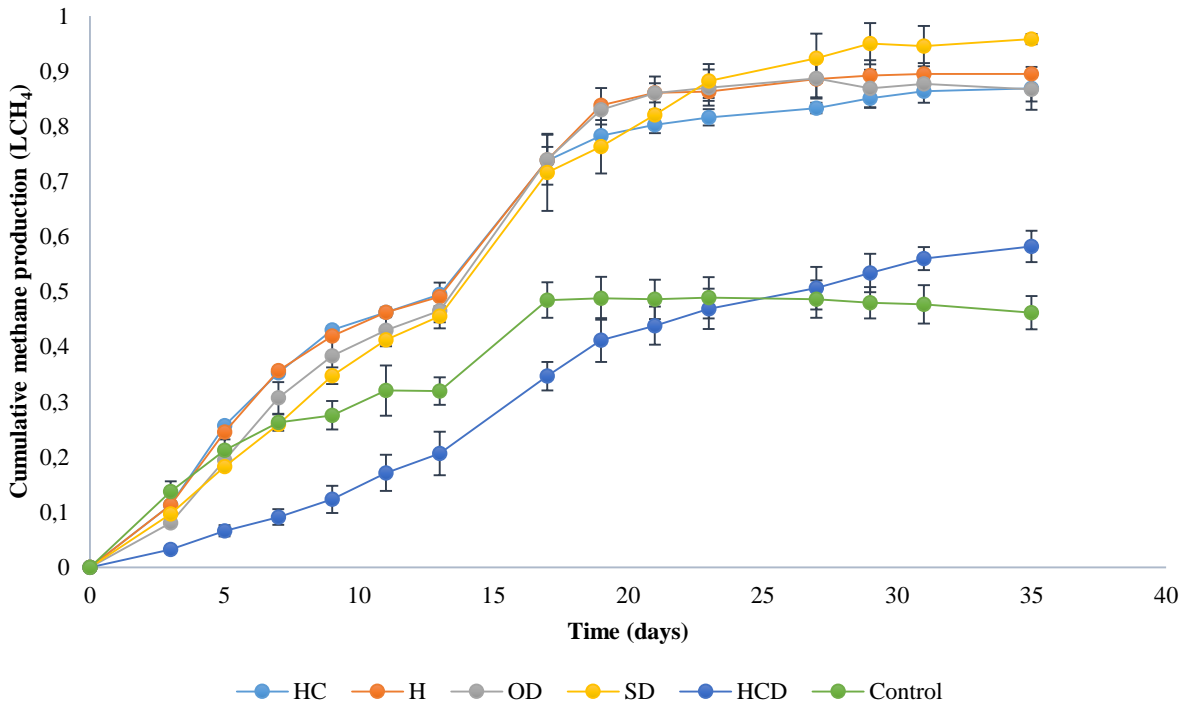


Figure 4.6: Comparison of five pre-treatment methods. Error bars represents the standard deviation ($n = 3$)

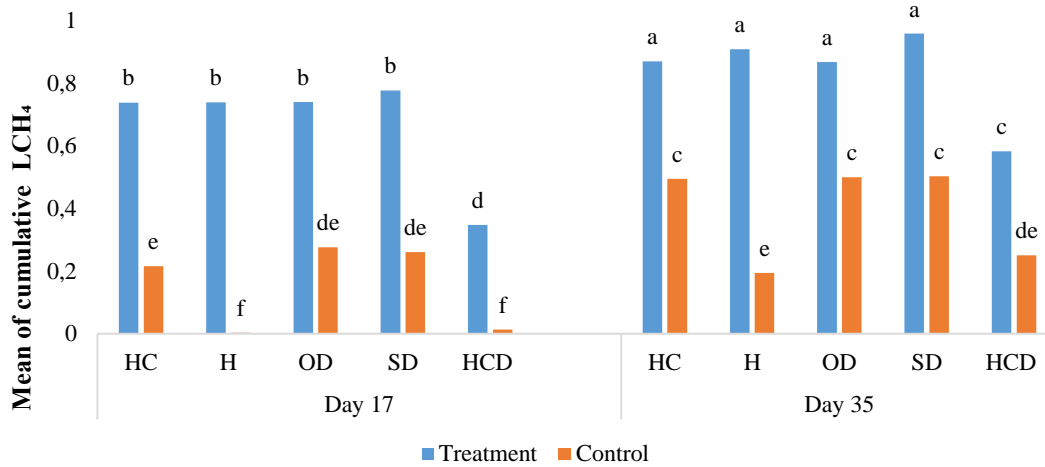


Figure 4.7: Mean of cumulative CH₄ production on day 17 and day 35. Similar letters on the bars represents no significant difference ($P > 0.05$), whereas different letters represents significant difference ($P < 0.05$).

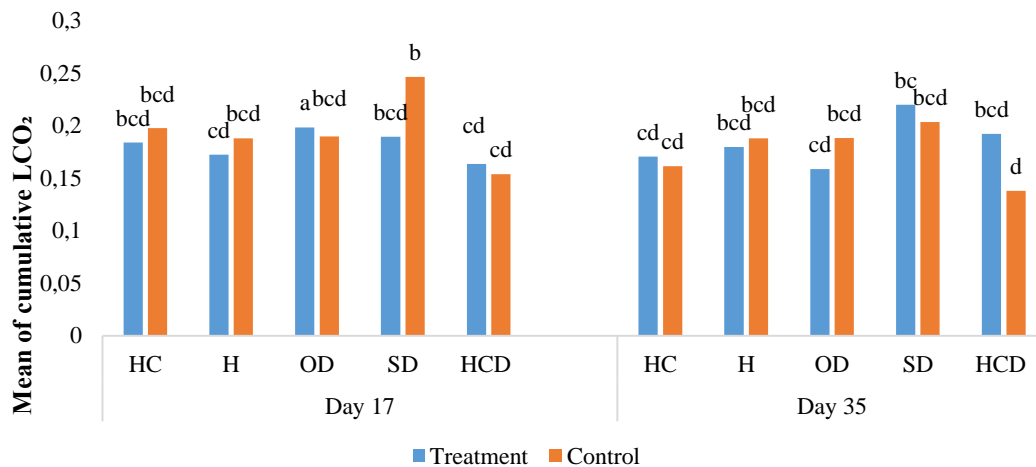


Figure 4.8: Mean of cumulative CO₂ production on day 17 and day 3. Similar letters on the bars represents no significant difference ($P > 0.05$), whereas different letters represents significant difference ($P < 0.05$).

Comparison of the VS before and after AD (Table 4.3) showed high substrate biodegradability from OD, with 9.87% of the VS reduced after AD while the least VS reduction was observed for the HCD pre-treatment (2.47%). The cumulative CH₄ produced (L) and the VS before AD were used to calculate the CH₄ yield (litres of CH₄ per gram volatile solids (LCH₄ gVS⁻¹)) (Table 4.4). The results showed OD to have the highest CH₄ yield of 1.78 L on day 17, while HCD showed the

lowest amount of 1.12 L on day 17. However, at the end of the BMP assay, SD had produced a highest CH₄ yield of 2.30 L.

As explained above, the cumulative CH₄ production (Figure 4.3), the amount of reduced VS after AD (Table 4.3) and the CH₄ yield (Table 4.4) revealed a different order in terms of the highest to the lowest biodegradation rates and CH₄ production. The cumulative CH₄ production followed this order: H, HC and OD produced the highest cumulative while SD and HCD produced the lowest on day 17. However, on day 35 the cumulative CH₄ production followed this order: SD was the highest followed by H, OD and HC while HCD was the lowest. The amount of reduced VS after AD at the end of Batch AD followed this order: OD reduced the highest VS, followed by H, SD, and HC while HCD reduced the lowest VS.

The CH₄ yield followed this order on day 17: OD produced the highest CH₄ yield, followed by SD, HC and H, while HCD produced the lowest CH₄ yield. However, on day 35, the cumulative CH₄ production followed this order: SD produced the highest CH₄ yield, followed by OD, H and HC while HCD produced the lowest amount of CH₄ yield. In the comparison of the highest to the lowest order as given above, it was observed that OD and HCD were the same for the three calculations, while the others differed.

Table 4.3: Percentage of volatile solids before and after anaerobic digestion

Samples	Volatile solids (% dry matter)		Degraded VS after AD (%)
	Before AD	After AD	
H	81.94	74.84	7.1
HC	79.62	74.53	5.09
OD	80.56	70.69	9.87
SD	80.68	74.59	6.09
HCD	74.07	71.60	2.47
Control	89.13	ND*	ND*

*Not determined

Table 4.4: Cumulative methane yield (L) per gram of volatile solids

Samples	CH ₄ yield		Volatile solids used up
	Day 17	Day 35	End
H	1430 ± 0.06	1700 ± 0.08	7.1
HC	1480 ± 0.08	1820 ± 0.11	5.09
OD	1780 ± 0.11	2100 ± 0.05	9.87
SD	1720 ± 0.06	2300 ± 0.06	6.09
HCD	670 ± 0.05	1120 ± 0.05	2.47
Control	1180 ± 0.08	1133 ± 0.07	ND*

*not determined, n = 3; ± standard deviation

4.3. Continuous AD assay

4.3.1 Process performance and stability

The process performance of the two semi-continuous anaerobic digesters was evaluated based on the biogas production and the quality of biogas measured by the amount of CH₄, while the stability of the process was monitored by measuring the FOS/TAC ratio and pH. The disturbance to the digesters was simulated by varied OLRs. The biogas produced, the FOS/TAC ratio and the pH were related to the microbial community present at each stage.

Stage 1: In both mono- and co-digestion, the cumulative biogas production increased slowly from 9 L and 7 L at the beginning of the stage to 16 L and 14 L at the end of the stage respectively (Figure 4.9 A). The average daily biogas production of 0.81 L and 0.78 L (Figure 4.9 B) for mono- and co-digestion was observed respectively. The biogas composition was monitored and a highest CH₄ content of 45.3% and 35% was observed while a CO₂ content of 41% and 27% of was observed in both mono- and co-digestion respectively (Figure 4.10). An average CH₄ yield (per gVS⁻¹) of 3.5 L for mono-digestion as compared to 2.2 CH₄ yield for co-digestion was recorded (Figure 4.11). The FOS/TAC ratio for mono-digestion was high at the beginning of the stage but decreased to an optimal range as the stage progressed (Figure 4.12). In co-digestion, the FOS/TAC

ratio was within the optimal range of between 0.4 to 0.6 at the beginning of the stage but decreased below optimal range as the stage progressed. In both digesters, the pH fluctuated throughout the stage, with co-digestion slightly higher than mono-digestion (Figure 4.13).

Stage 2: In this stage, differences were observed between the two digesters. The cumulative biogas production greatly increased in mono-digestion (from 17 L at the beginning to 31 L at the end), while, in co-digestion, it remained within 15.1 L to 15.8 L (Figure 4.9 A). A similar trend was observed with the average daily biogas production (Figure 4.9 B) with the mono-digestion absolute biogas increasing to 1.53 L every second day in comparison to stage 1. However, in co-digestion, the average daily biogas production drastically reduced to 0.06 L. In mono-digestion, the highest recording of 44% for CH₄ and CO₂ was observed while, in co-digestion, this decreased as compared to stage 1 to 27% of CH₄ and 28% of CO₂ (Figure 4.10). In mono-digestion, the average CH₄ yield increased to 7.8 L while, for co-digestion, it increased slightly to 2.5 L (Figure 4.11). The FOS/TAC ratio remained in the range of 0.4 to 0.5 for mono-digestion and 0.1 to 0.2 for co-digestion (Figure 4.12). In addition, in this stage, the pH fluctuated but was slightly higher than in stage 1 although co-digestion was still slightly higher as compared to mono-digestion (Figure 4.13).

Stage 3: In mono-digestion, cumulative biogas production continued to increase (from 35 L to 52 L) (Figure 4.9 A). The biogas content (CH₄ and CO₂) remained approximately similar to that in stage 2 while the CH₄ yield slightly decreased to 7.4 L (Figure 4.11). Co-digestion resulted in process failure, where the cumulative biogas, average daily biogas production and CH₄ production continuously decreased until day 77 when no biogas production was recorded (Figure 4.9, 4.10 and 4.11). However, 2 days later (day 80), biogas production in negligible quantity was observed.

Overall, the comparison of each stage of the two digesters showed differences in the biogas, CH₄ and CO₂ produced (Table 4.5). The comparison of the two digesters showed that mono-digestion performed better in all the stages than co-digestion. Moreover, stage 1 for mono-digestion demonstrated the highest amount of the total CH₄ produced in the 21 days. On the other hand, stage 1 of co-digestion slightly produced a higher total biogas, whereas the CH₄ content was lower in comparison to mono-digestion. Furthermore, stage 2 and 3 for both digesters resulted in slightly higher amounts of CO₂ produced in comparison to the amount of CH₄ produced.

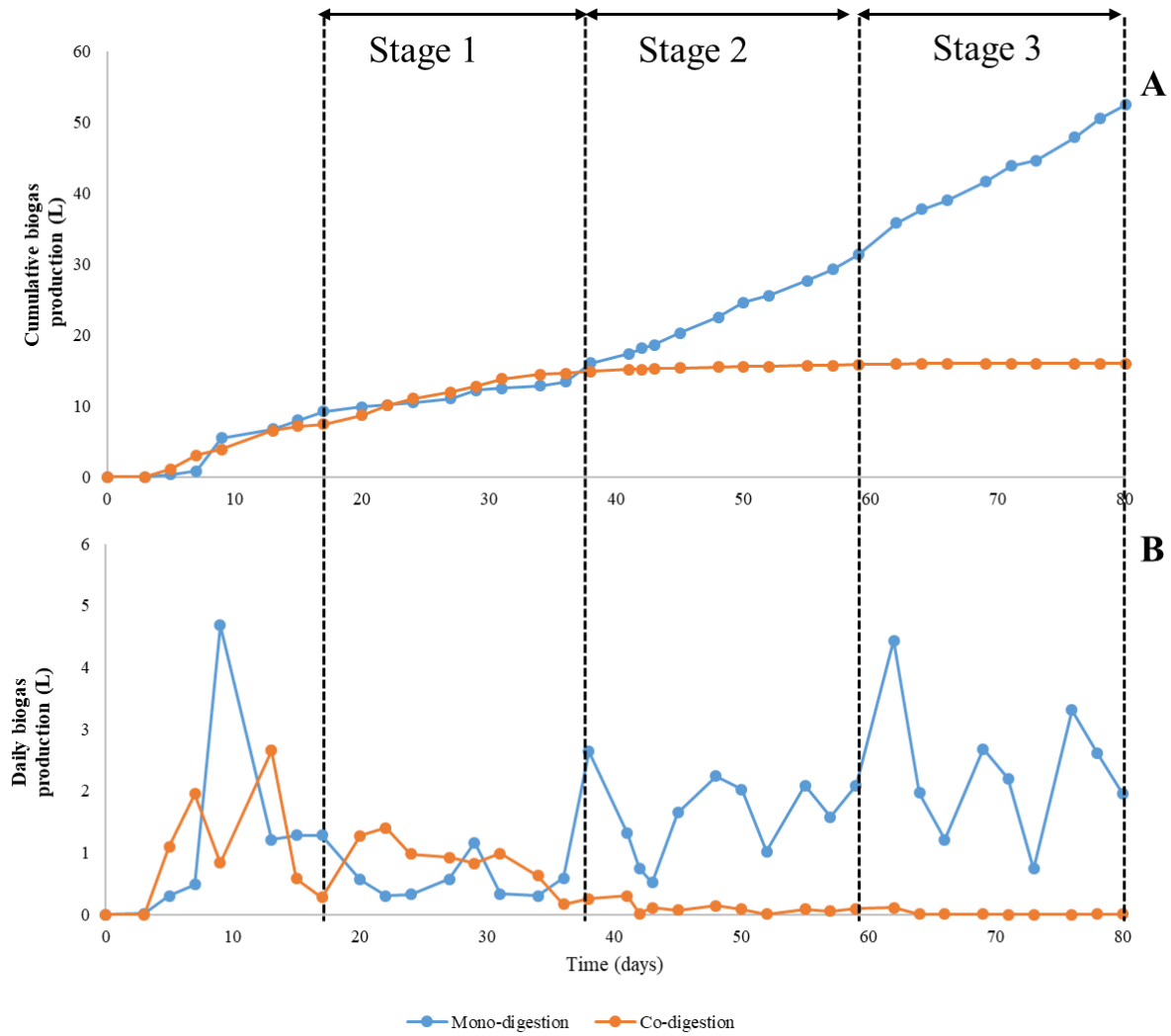


Figure 4.9: Cumulative biogas (A) and daily biogas (B) production for mono- and co-digestion. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

Table 4.5: Total biogas, methane and carbon dioxide produced at each stage for mono and co-digestion.

	Mono-digestion	Co-digestion
Stage 1		
Total biogas (L)	96.5	97.9
CH ₄ (L)	41.3 (42.8%)	28.5 (29.1%)
CO ₂ (L)	34.7 (35.9)	23.0 (23.5%)
Stage 2		
Total biogas (L)	138.9	56.9
CH ₄ (L)	55.2 (39.7%)	8.0 (14.1%)
CO ₂ (L)	58.7 (42.3%)	9.3 (16.3)
Stage 3		
Total biogas (L)	254.4	56.6
CH ₄ (L)	101.2 (39.8%)	9.5 (16.8%)
CO ₂ (L)	101.8 (40%)	9.3 (16.4%)

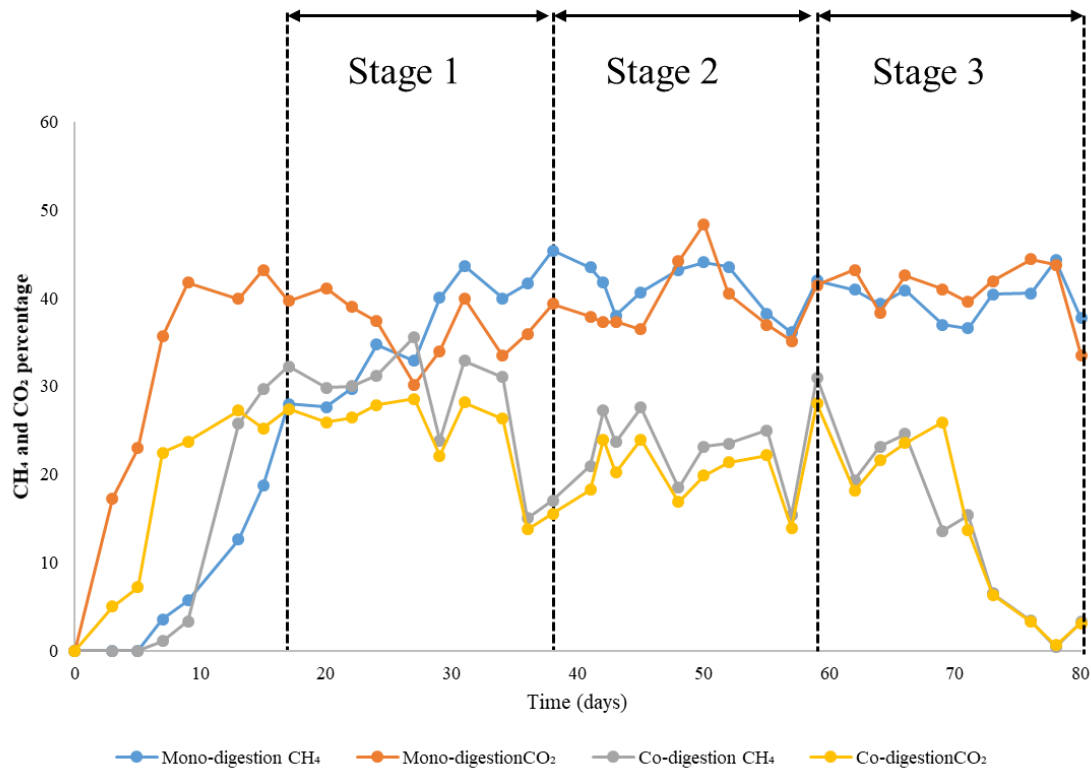


Figure 4.10: Cumulative CH₄ and CO₂ production for mono- and co-digestion. Stage 1- feeding once a week with 2%TS, stage 2- feeding twice a week with 2% TS and stage 3- feeding once a week with 4% TS

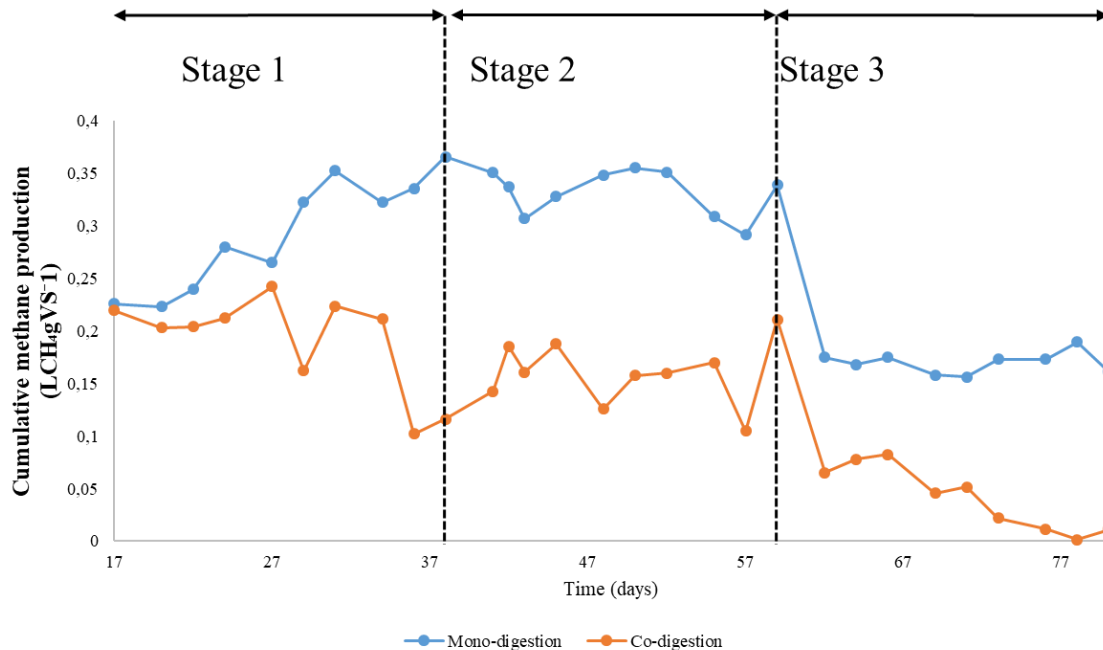


Figure 4.11: CH_4 yield per gram volatile solids from mono- and co-digestion. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

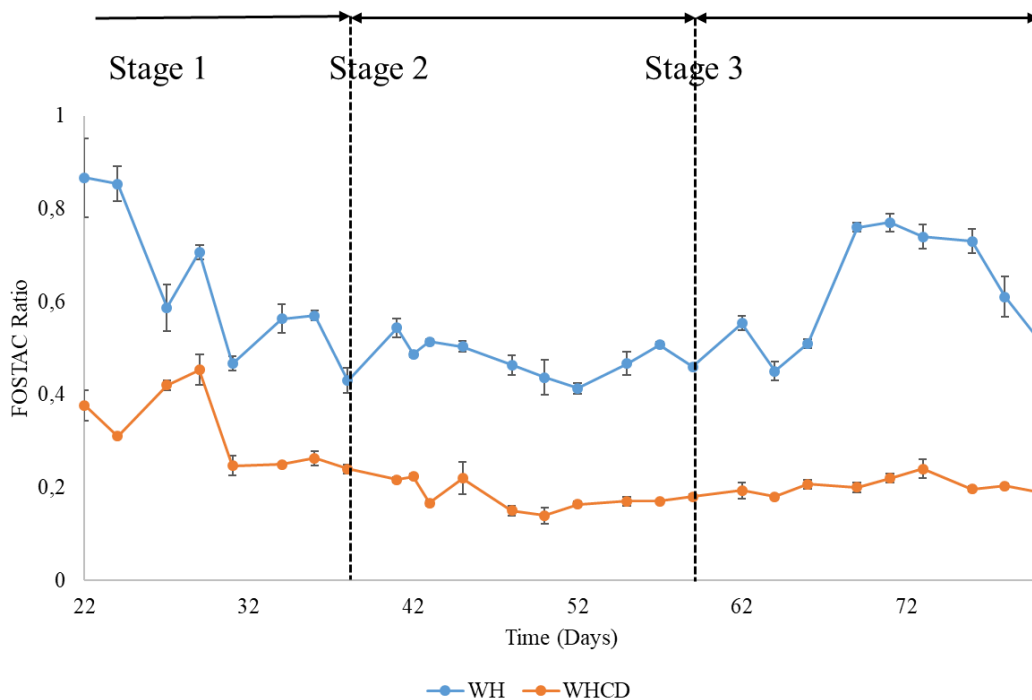


Figure 4.12: FOS/TAC ratio from mono- and co-digestion. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

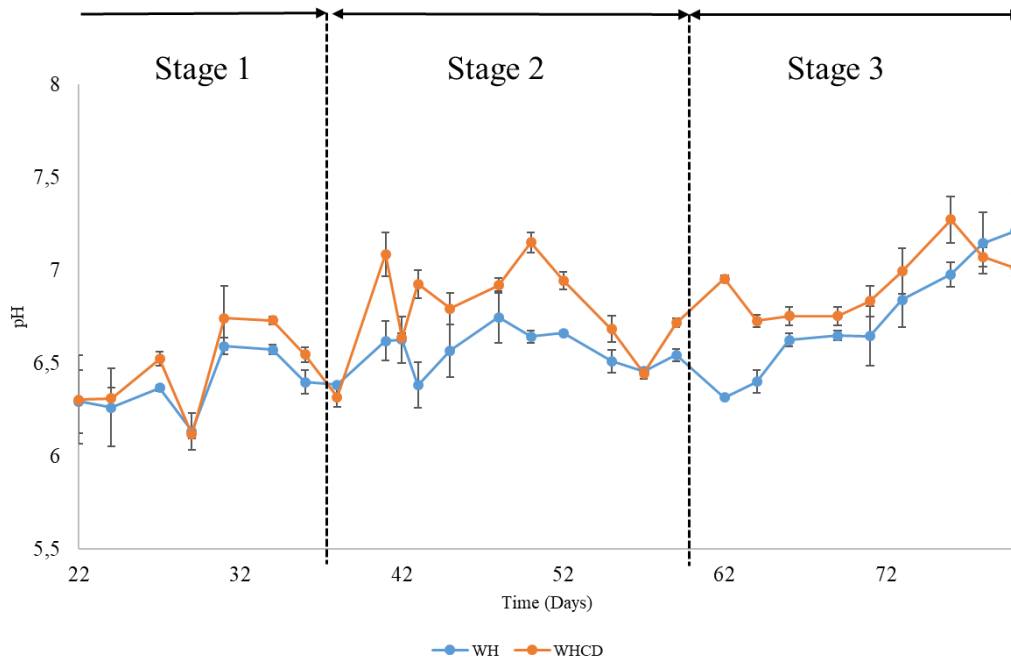


Figure 4.13: pH of the two digesters. Stage 1 – feeding once a week, stage 2 – feeding twice a week and stage 3 – feeding once a week with increased VS

4.4. Microbial analysis

4.4.1 Metabolic genes amplification

Metabolic genes involved in acetogenesis (FTHFS) and methanogenesis: *mcrA*- hydrogenotrophic pathway and ACAS- acetoclastic pathway were amplified for the same samples used for NGS sequencing (Figure 4.14). Figure 4.14 A shows that two samples from the co-digestion, sample 3DA (end of stage 1) and 1DB (beginning of stage 2), did not contain the metabolic gene FTFHS, while all the mono-digestion samples contained the gene. As for methanogenesis, all the samples (both mono- and co-digestion) contained the gene *mcrA*. More samples from co-digestion contained the gene ACAS (Figure 4.14 B and C respectively) in comparison to mono-digestion. Selected genes were identified in order to confirm identity. The phylogenetic tree was constructed with the selected amplified genes, which formed clusters with their relatives obtained from NCBI (Appendix A4.1).

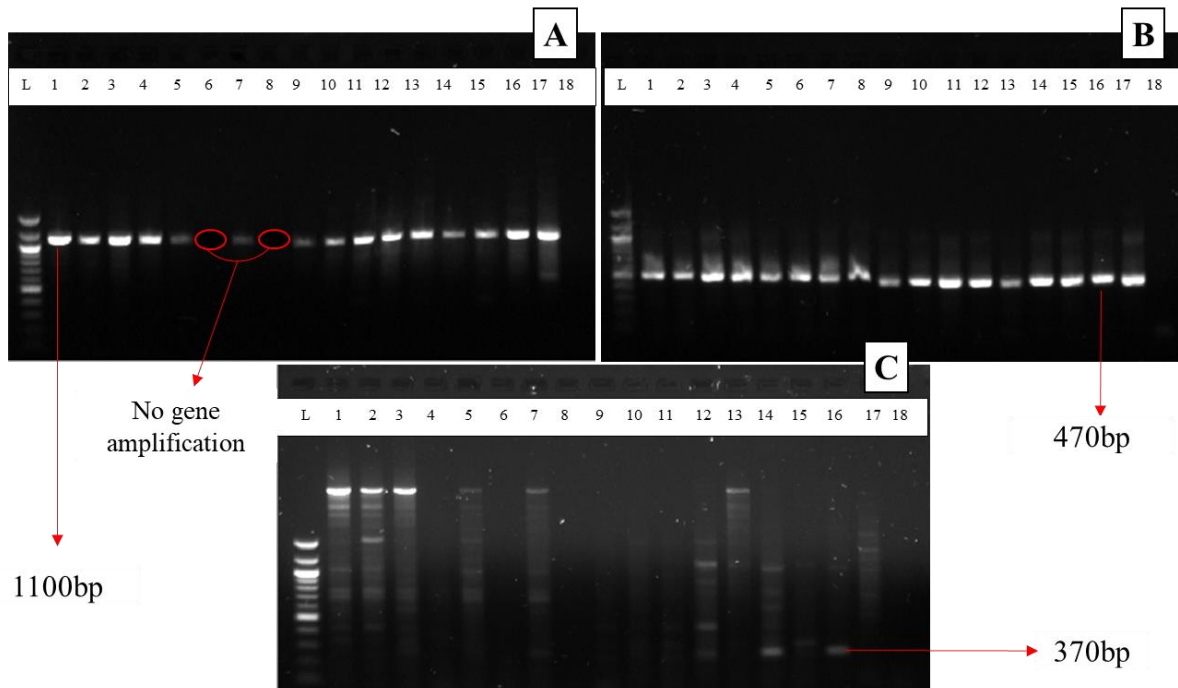


Figure 4.14: Metabolic gene amplifications for mono- and co-digestion. A-FTFHS, B-*mcrA* and C- ACAS genes. 1-1WA, 2-1DA, 3-2WA, 4-2DA, 5-3WA, 6-3DA, 7-1WB, 8-1DB, 9-SWB, 10-2DB, 11-3WB, 12-3DB, 13-2WC, 14-2DC, 15-3WC, 16-3DC, 17-Inoculum, 18-negative

4.5. Description of bacterial diversity and shifts in community structure in each stage

A total of 25 276 operational taxonomic units (OTUs) were obtained from both mono- and co-digestion samples. The OTUs were obtained after rarefaction at an even depth of 74533 per sample. The rarefied OTU table was used for computing the alpha and beta diversity

4.5.1 Alpha diversity indices of bacteria.

The alpha diversity, which compares the number of species within each sample, was measured. The alpha diversity indices for bacterial communities fluctuated between the stages (Table 4.6). However, stage 2 showed high bacterial diversity and evenness, mainly at the early and mid-time points of some stages for mono-digestion while for co-digestion was the mid and end time points. A decrease in observed OTUs for both mono- and co-digestion at the end time point of stages 2 and 3 was observed. In addition, for mono-digestion, a decrease in bacterial diversity was also observed in Shannon and Chao1. A comparison of the overall bacterial diversity in mono- and co-digestion showed a higher diversity in co-digestion.

4.5.2 Beta diversity of bacteria

The beta diversity distances measures between the bacterial samples for both mono- and co-digestion were evaluated using Principal Coordinate Analysis (PCoA). Beta diversity measures compare the number of species shared between samples. It is divided into two categories, namely, weighted, known as quantitative, and unweighted, known as qualitative. The weighted category compares the number of species based on the relative abundance of each type of organism while the unweighted compares the microbial community composition based on either presence or absence.

The PCoA plots did not show distinct clustering of the samples collected from the early, mid or late sampling time points for both digesters (Figure 4.15 A and B). However, strong similarities during the early and late sampling time points were observed for co-digestion (defined by the overlap between the clusters). Minimal overlapping was observed between the other samples for both digesters. Although the overlapping was less, the sample from the late time point mono-digestion overlapped with almost all samples, except for the samples obtained from the mid mono-digestion for both Figures. There was no significant difference for weighted PCoA (PERMANOVA $R^2 = 0.37$, pseudo- $F = 1.2$ $P > 0.21$, ANOSIM $R = 0.12$, $P > 0.15$). Significant difference for unweighted PCoA was observed only from PERMANOVA ($R^2 = 0.42$, pseudo- $F = 1.5$ $P < 0.03$) while ANOSIM ($R = 0.19$, $P > 0.07$) was not.

A heat map of the bacterial communities based on the abundance of the genera within a hierarchical cluster based on Bray-Curtis was generated. Dissimilar patterns from the early, mid and late sampling time points for both mono-digestion and co-digestion were observed (Figure 4.16). In addition, the clusters from both digesters differed from the inoculum.

Table 4.6: Alpha diversity indices of bacteria in mono- and co-digestion. E – early, M – mid and L – late.

Sample	Observed OTUs		Shannon index		Simpson index		Chao1	
Inoculum	973		4.48		34.18		1208.08	
	Mono	Co	Mono	Co	Mono	Co	Mono	Co
Stage 1 (E)	1284	1205	3.52	3.29	5.55	6.44	1539.03	1480.77
Stage 2 (E)	1372	1541	3.95	4.33	8.18	23.59	1697.57	2032.02
Stage 3 (E)	1684	1625	4.46	4.08	24.27	13.57	2089.23	2027.57
Stage 1 (M)	1502	1483	4.39	4.16	22.19	16.54	1878.78	2014.43
Stage 2 (M)	1555	1751	3.91	4.30	11.82	14.77	2026.00	2187.50
Stage 3 (M)	1536	1600	4.10	4.29	16.86	20.89	2007.16	2133.27
Stage 1 (L)	1511	1540	4.02	4.35	14.50	23.46	1912.76	1915.11
Stage 2 (L)	1276	1479	4.09	4.55	20.61	30.47	1762.16	1856.40
Stage 3 (L)	1225	1394	3.91	4.16	17.32	18.94	1694.01	1859.41

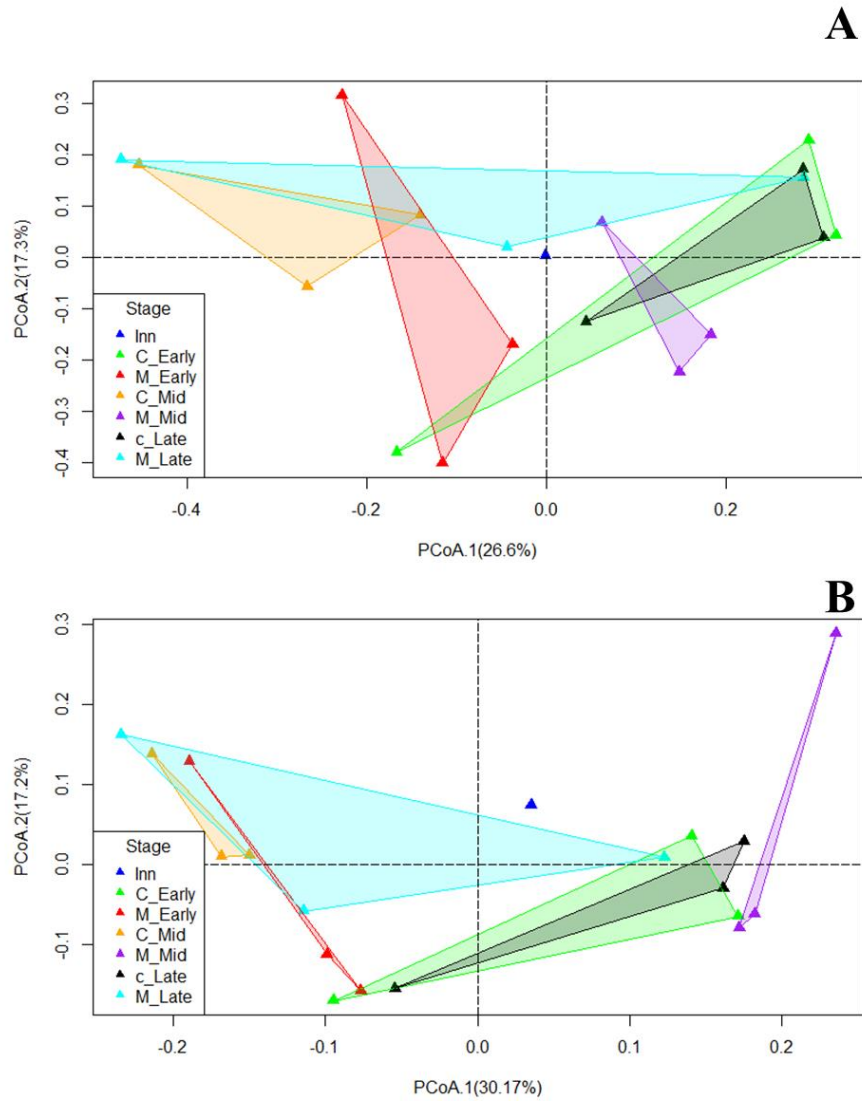


Figure 4.15: Weighted and (B) unweighted Bray-Curtis measures of beta-diversity visualised using principal coordinate analysis (PCoA) for the comparison of bacterial diversity at each sampling time for both mono- and co-digestion. M represents samples from mono-digestion and C samples from co-digestion

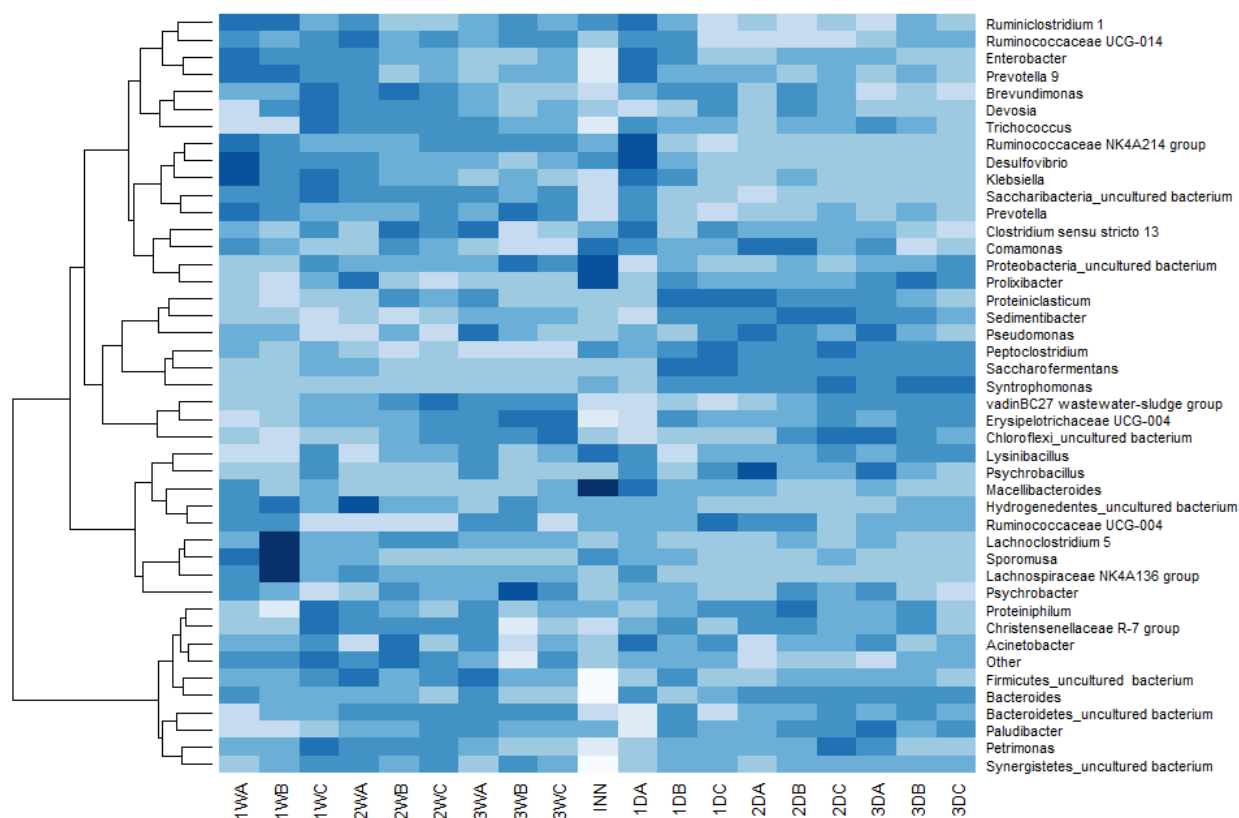


Figure 4.16: Heat map of the bacterial communities based on the abundance of genus from mono- and co-digestion

4.5.3 Taxonomic diversity of bacterial community on mono- and co-digestion

The relative abundance of bacteria in the different stages was analysed from phylum to genus levels comprising at least 1% in at least one sample (Figures 4.17 A and B, 4.18 and 4.19 respectively). Among the bacteria, the phyla Bacteroidetes, Proteobacteria, Firmicutes and Chloroflexi and Parcubacteria were found to be abundant (Campanaro et al., 2018; Yi et al., 2014). Although dominated by these phyla, each digester had its own unique bacterial community composition. The phyla Bacteroidetes was the most dominant in most of the samples and consisted of the order Bacteroidales. This order consisted of families such as Bacteroidaceae and Porphyromonadaceae while, from these two families, the genera *Bacteroides*, *Proteiniphilum*, *Petrimonas*, *Paludibacter* and *Prevotella*, were observed. For the phylum Proteobacteria, Moraxellaceae and Pseudomonadaceae were the two families observed. The analysis of the phylum revealed *Acinetobacter* and *Pseudomonas* to be the dominant genera in some of the samples.

Early: The genus *Bacteroides* (47.6 and 38.5% respectively) dominated the beginning of stage 1 and stage 2 in mono-digestion, while *Bacteroides* and *Acinetobacter* dominated stage 3 (21.4% and 20.1%). In co-digestion, this genus was abundant but not dominant (abundance indicates the prevalence of >1%) at the beginning of stages 2 and 3. The beginning of stages 2 and 3 showed clear differences in the community composition structure. The beginning of stage 1 was dominated by *Acinetobacter* and *Bacteroides* (36.8 and 33.9% respectively), while stage 2 was dominated by *Bacteroides*, *Petrimonas*, Bacteroidetes and Firmicutes uncultured bacterium, *Christensenellaceae* R-7 group, *Paludibacter*, *Proteiniclasticum* and *Proteiniphilum* (from most abundant to less abundant (18.1–4.1%)) while stage 3 was dominated by *Acinetobacter*, *Petrimonas*, *Christensenellaceae*, *Proteiniclasticum* (ranging from 31.7–4.2%).

Mid: As the stages progressed, more abundant genera became common in all the stages. These include the genera *Bacteroides*, *Proteiniphilum*, *Acinetobacter*, *Christensenellaceae* R-7 group, *Petrimonas*, *Paludibacter* and uncultured bacterium from the phyla Bacteroidetes and Chloroflexi, Although differences were observed, in mono-digestion, mid sampling point of stage 1 was dominated mainly by *Bacteroides* (25%) whereas stages 2 and 3 were dominated by *Acinetobacter* (32.4% and 18.4% respectively). High dominant numbers of genera were observed in stages 1 and 3.

In co-digestion, the genus *Pseudomonas*, *Petrimonas*, *Acinetobacter* and *Christensenellaceae* R-7 group dominated the beginning of stage 1 (25%, 16.1%, 15.7% and 12.7% respectively), while stage 2 was dominated by *Acinetobacter*, *Petrimonas* and *Proteiniphilum*. In addition to the genera in stage 2, stage 3 also included genera *Paludibacter* and Chloroflexi uncultured bacterium as the dominant genera.

End: At the end of all the stages, commonly shared genera we also observed although in varying degrees. The genera *Proteiniphilum* and *Paludibacter* were approximately similar in all the stages of mono-digestion. *Acinetobacter*, *Proteiniphilum* and *Paludibacter* (29.1%, 14.8%, 10.1% respectively) dominated the end of stage 1 while *Paludibacter*, *Proteiniphilum* and *Bacteroides* (19.1%, 16.5% and 14.2% respectively) dominated stage 2 and *Acinetobacter*, *Paludibacter* and *Bacteroides* (19.8%, 19.7 and 10.3% respectively) dominated stage 3. In co-digestion, the end of stage 1 did not contain the genus *Proteiniphilum* that was observed as abundant in stages 2 and 3 (20% and 29.6% respectively). In addition to *Proteiniphilum*, *Acinetobacter* and *Pseudomonas*

(21.3% and 11.75% respectively) dominated stage 1 while *Acinetobacter* and *Petrimonas* (10.9% and 10% respectively) dominated stage 2.

Overall, the genera that were found to be distinct to each stage in mono-digestion (between 4 to 6%) included *Provetella*, *Enterobacter* and *Ruminococcaceae* UCG-014 in stage 1, *Lachnospiraceae* NK4A136 group, *Ruminiclostridium* and *Erysipelotrichaceae* UCG-004 in stage 2 and *Petrimonas* in stage 3. In co-digestion, *Pseudomonas* was found only at the mid and end sampling points of stage 1.

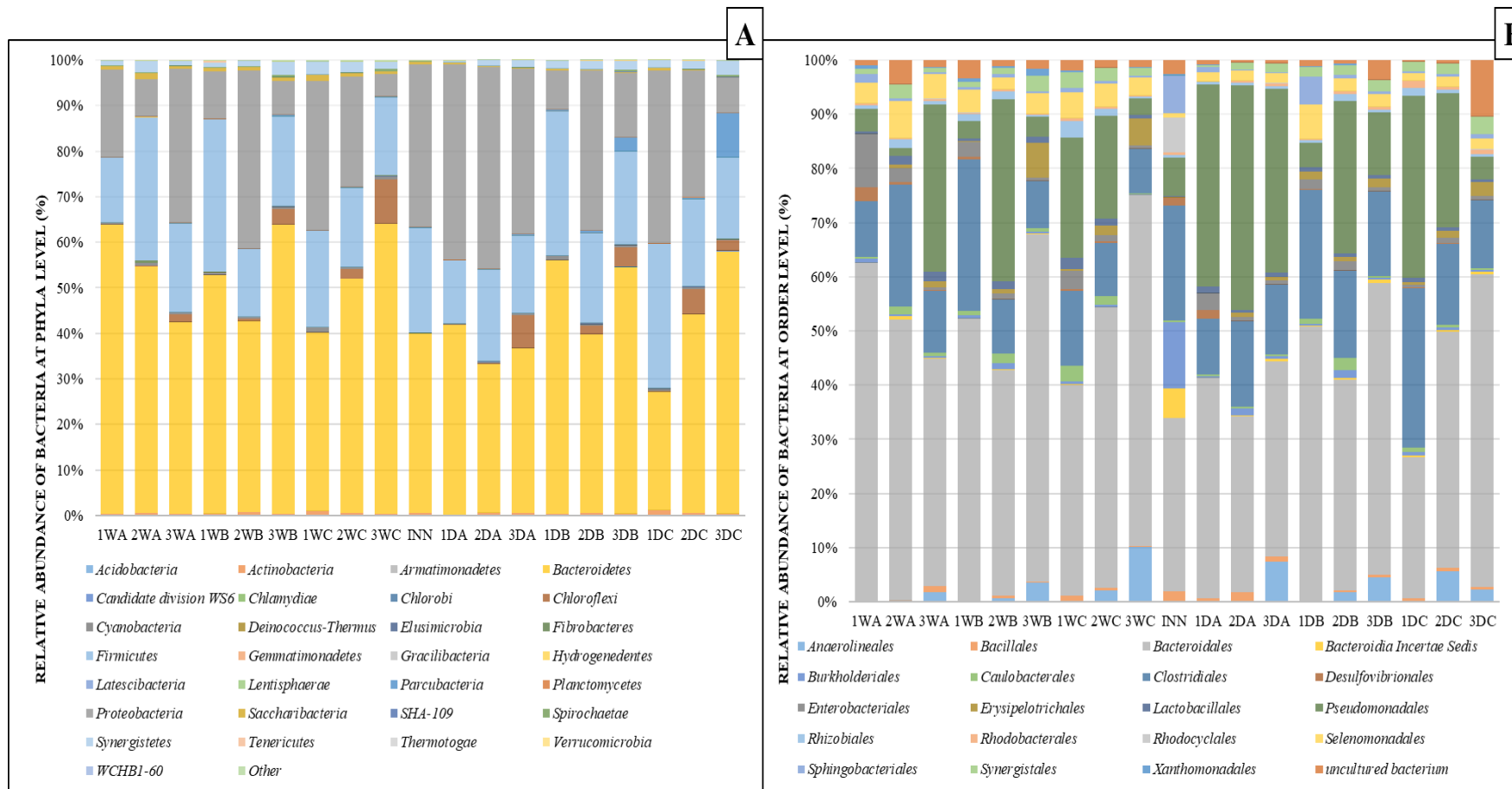


Figure 4.17: Relative abundance of bacteria at phyla (A) and order (B) level for mono- (W) and co-digestion (D). A – stage 1, B – stage 2 and C – stage 3, 1 – beginning, 2 – mid and 3 – end of stage and INN – inoculum

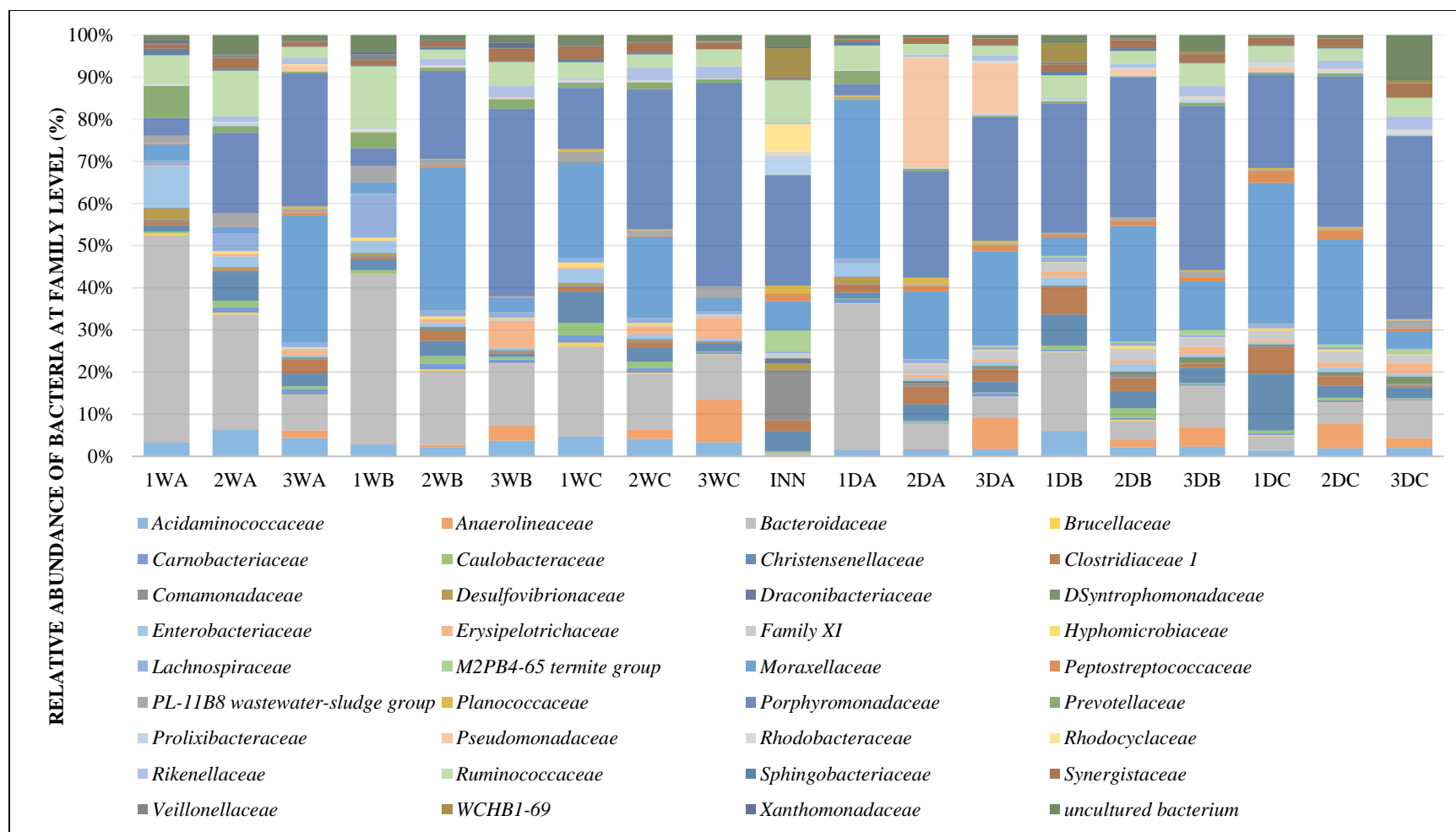


Figure 4.18: Relative abundance of bacteria family level for mono- (W) and co-digestion (D). A – stage 1, B – stage 2 and C – stage 3, 1 – beginning, 2 – mid and 3 – end of stage and INN – inoculum

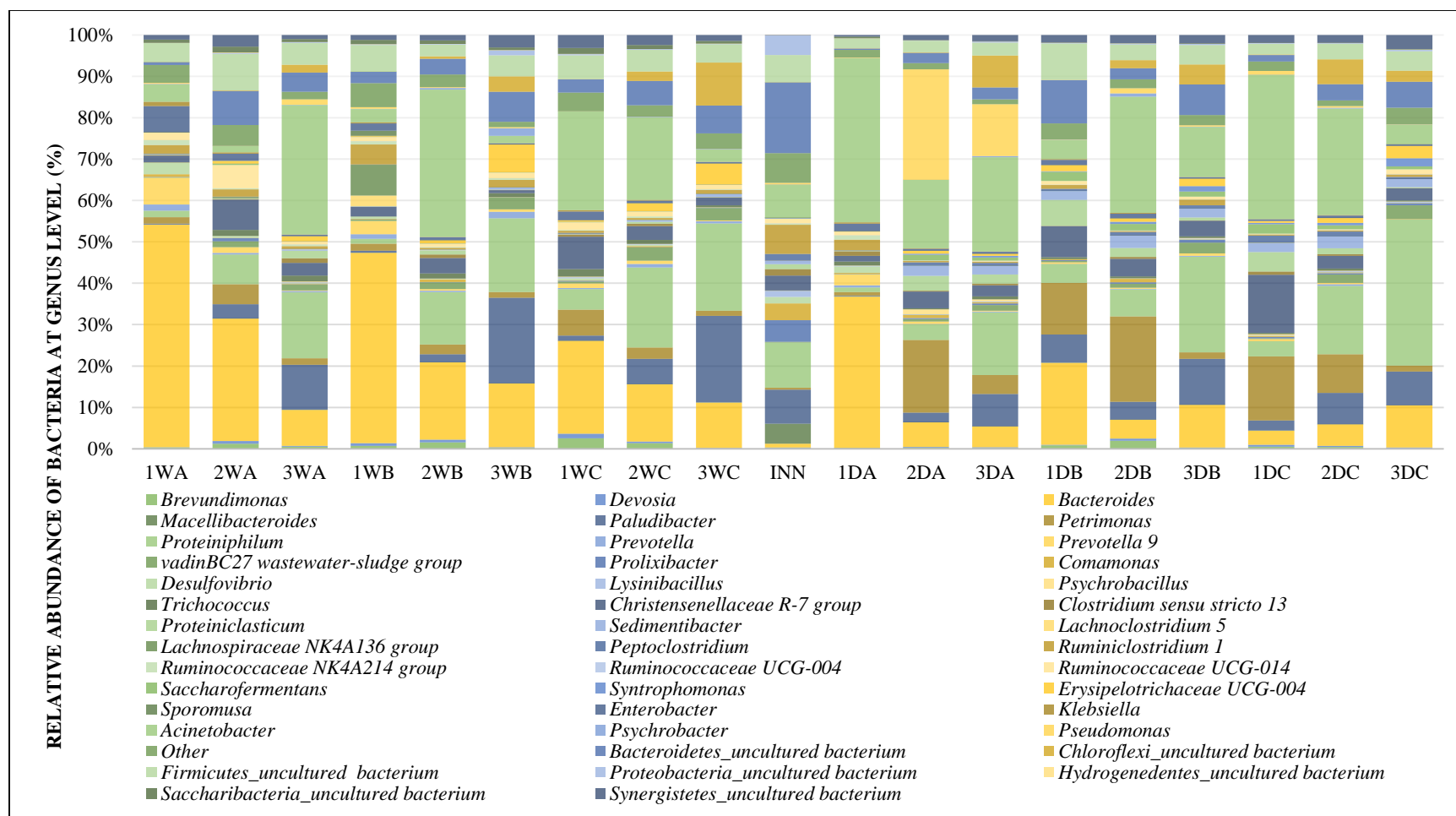


Figure 4.19: Relative abundance of bacteria at genus level for mono- (W) and co-digestion (D). A – stage 1, B – stage 2 and C – stage 3, 1 – beginning, 2 – mid and 3 – end of stage and INN – inoculum

4.6. Description of archaeal diversity and shifts in community structure in each stage

A total of 342 operational taxonomic units (OTUs) were obtained from both the mono- and co-digestion samples. The OTUs were obtained after rarefaction at an even depth of 74533 per sample while the rarefied OTU table was used for computing the alpha and beta diversity.

4.6.1 Alpha diversity of archaea

The alpha diversity indices of the archaeal community were much lower than that of the bacteria (Table 4.7). The comparison of stages 2 and 3 to stage 1 mono-digestion showed a fluctuation in the alpha diversity indices at the mid and some of the end sampling points. In co-digestion, the comparison of stages 2 and 3 to stage 1 showed a decrease in diversity when the Simpson and Shannon indices were computed at the early and mid sampling points of stages. On the other hand, Chao1 and the observed OTUs showed an increase in diversity at the early and mid sampling points of all the stages.

4.6.2 Beta diversity of archaea

The weighted and unweighted beta diversity distances measures between the archaeal samples for both mono- and co-digestion were evaluated using Principal Coordinate Analysis (PCoA). The PCoA plots did not show distinct clustering in most of the samples (Figure 4.20 A and B) while strong similarities between mono- and co-digestion mid and early sampling points respectively were observed for both the weighted and unweighted. Minimal overlapping was observed between other samples for both the digesters. There was no significant difference for weighted PCoA (PERMANOVA $R^2 = 0.42$, pseudo- $F = 1.5$ and $P > 0.18$, ANOSIM $R = 0.0.15$, $P > 0.20$) whereas significant difference for the unweighted PCoA was observed (PERMANOVA $R^2 = 0.48$, pseudo- $F = 1.8$ $P < 0.04$ and ANOSIM $R = 0.23$, $P < 0.04$).

A heat map of the archaeal communities based on the abundance of the genera within a hierarchical cluster based on Bray-Curtis was generated. Similar patterns from the early, mid and late sampling points for both mono-digestion and co-digestion were observed in comparison to bacterial heat map (Figure 4.21). Similar to the bacterial PCoA, the clusters from both digester were very different to the inoculum.

Table 4.7: Alpha diversity indices of archaea in mono- and co-digestion. E – early, M – mid and L – late.

Sample	Observed OTUs		Shannon index		Simpson index		Chao1	
Inoculum	30		2.6		8.76		33.00	
	Mono	Co	Mono	Co	Mono	Co	Mono	Co
Stage 1 (E)	19	13	1.74	1.75	3.45	3.35	24	13
Stage 2 (E)	21	18	2.02	1.75	4.99	3.62	23	21.33
Stage 3 (E)	19	26	2.01	1.22	5.01	1.88	22.33	35
Stage 1 (M)	19	19	2.14	1.19	5.39	1.81	19.75	22.75
Stage 2 (M)	17	19	2.23	1.17	6.74	1.77	17.33	24.25
Stage 3 (M)	20	23	2.22	1.19	6.59	1.81	20.38	24
Stage 1 (L)	19	19	2.24	1.03	6.95	1.65	26	26
Stage 2 (L)	11	24	1.08	1.39	1.79	2.11	14.33	25.25
Stage 3 (L)	22	16	2.02	1.4	4.17	2.34	23.5	23.5

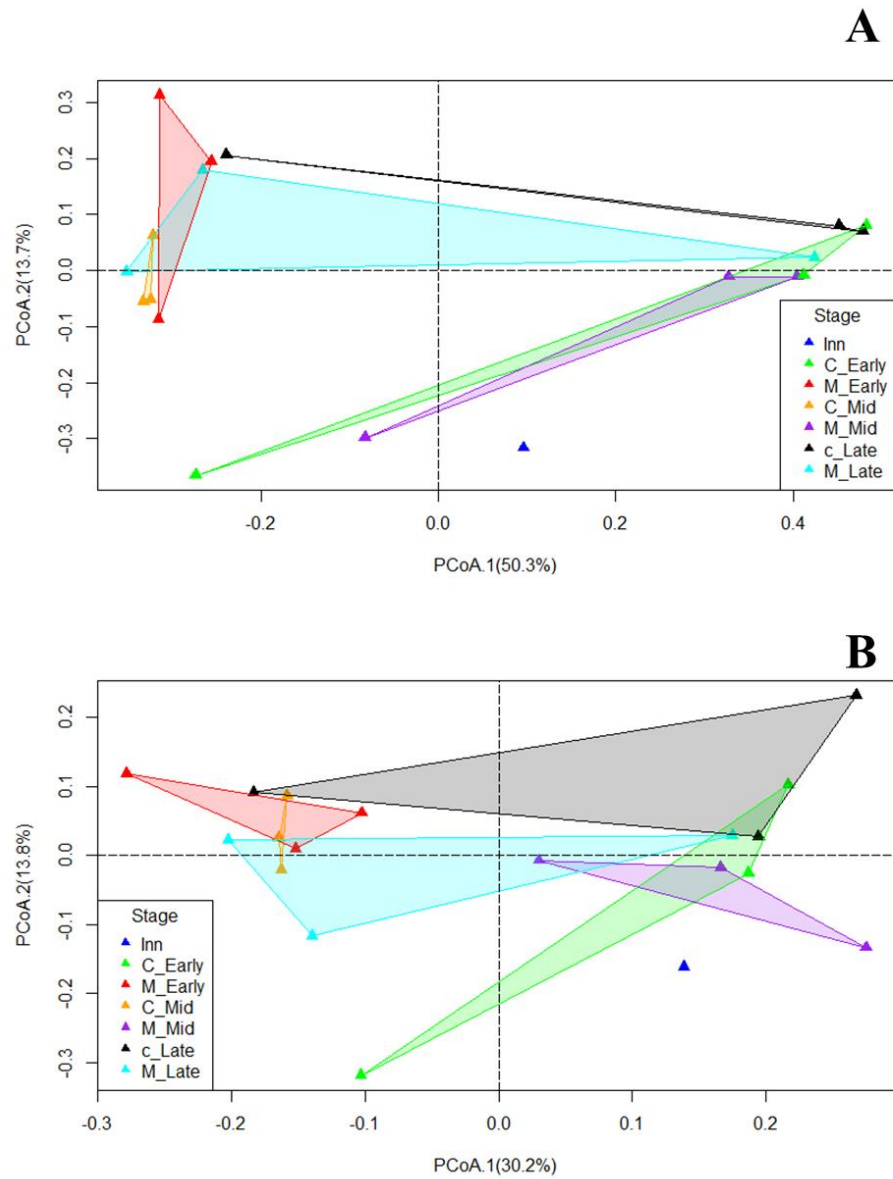


Figure 4.20: (A) Weighted and (B) unweighted Bray-Curtis measures of beta-diversity visualised using principal coordinate analysis (PCoA) for the comparison of the archaeal diversity at each sampling time for both mono- and co-digestion. M represents samples from mono-digestion and C samples from co-digestion

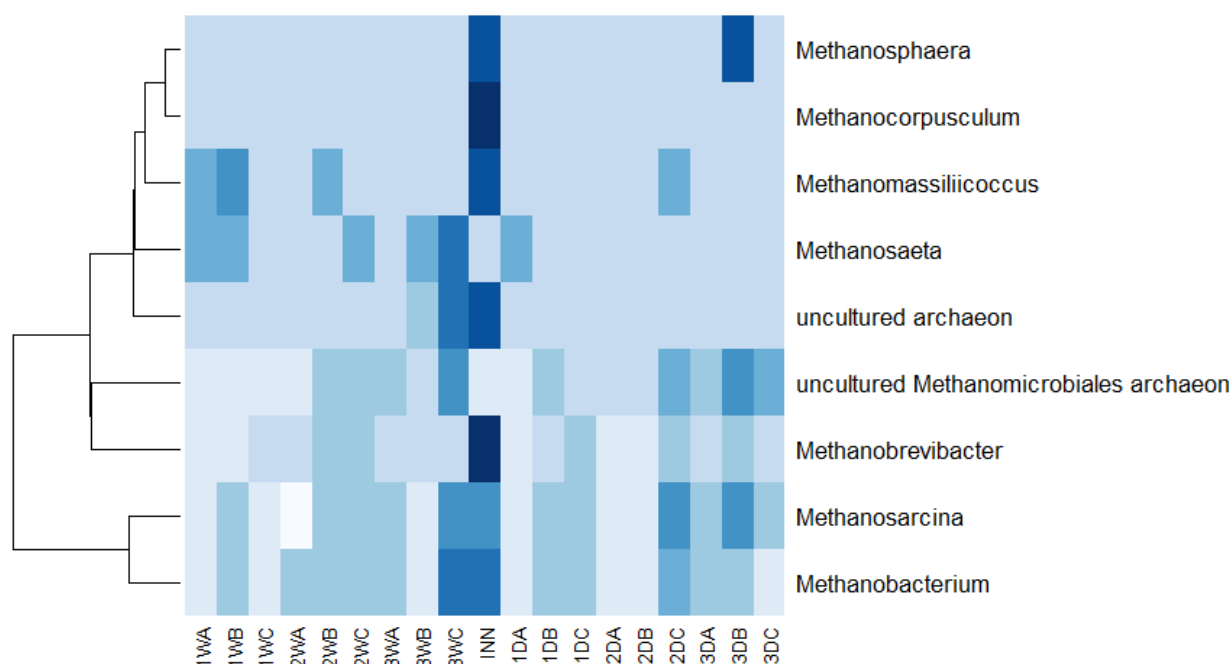


Figure 4.21: Heat map of the archaeal communities based on the abundance of genus from mono- and co-digestion

4.6.3 Taxonomic diversity of bacterial community on mono- and co-digestion

The relative abundance of archaea in the different stages was also analysed from the phylum to genus levels (Figures 4.22 A and B, 4.23 and 4.24 respectively). All the stages of mono- and co-digestion were dominated by the phyla Euryarchaeota, affiliated mainly with the order Methanosarcinales and Methanobacteriales. The two orders, composed of *Methanosarcina* and *Methanobacterium* respectively, were observed to be abundant in all the samples while *Methanobrevibacter* and uncultured *Methanomicrobioles* genera were less abundant.

In mono-digestion, the genera *Methanobacterium* and *Methanosarcina* were abundant in all the stages, with *Methanosarcina* slightly more abundant in most of the samples. However, *Methanobacterium* (87.1%) dominated mainly the end of stage 3. It was also observed that at the mid sampling point of all the stages the *Methanobacterium* decreased while *Methanosarcina* increased. Moreover, at the end of the stages, *Methanobacterium* increased (up to 87% in stage 2) while *Methanosarcina* decreased. In co-digestion, except for the beginning of stage 2, both genera were dominant although the genus *Methanosarcina* was more dominant (up to 95%) than *Methanobacterium* in all the stages.

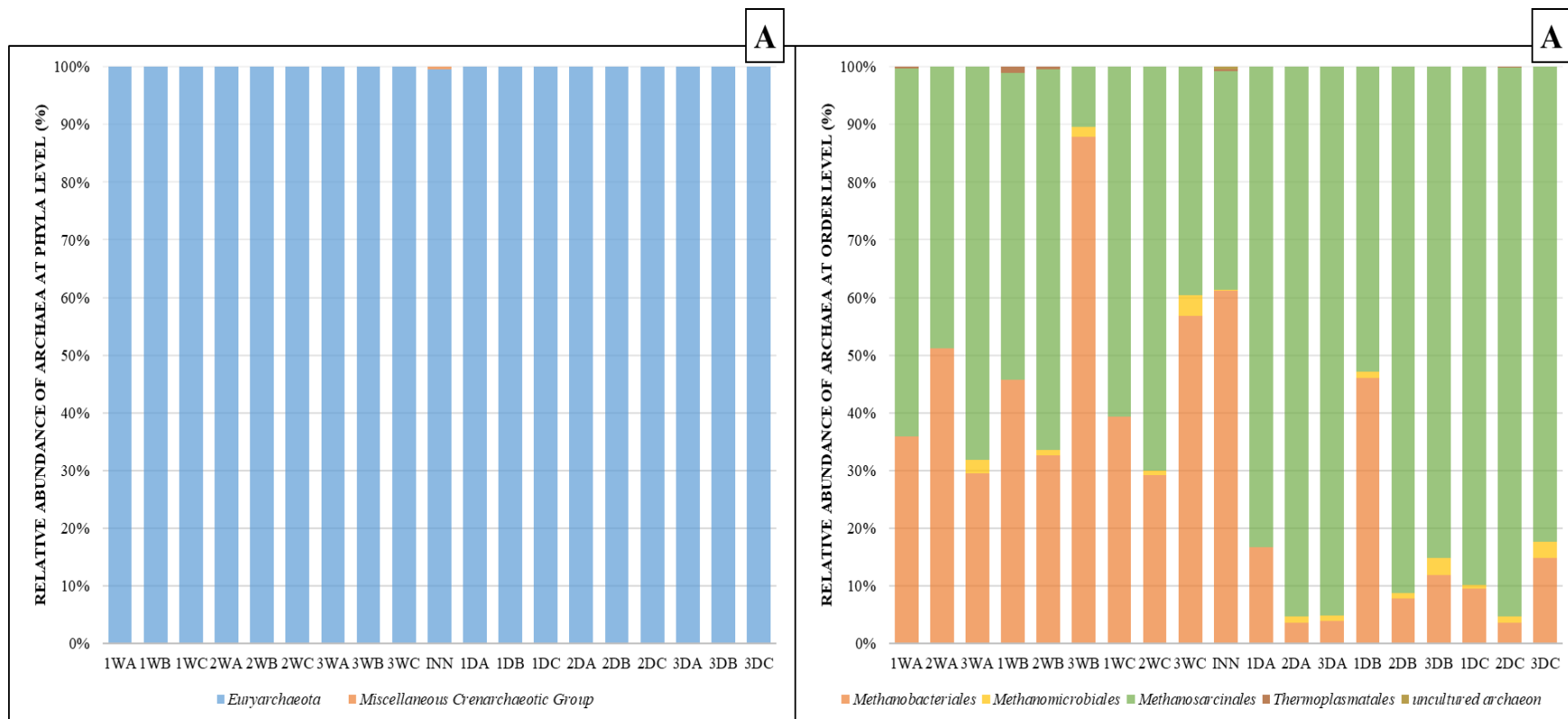


Figure 4.22: Relative abundance of archaea at phyla (A) and order (B) level for mono- (W) and co-digestion (D). A – stage 1, B – stage 2 and C – stage 3, 1 – beginning, 2 – mid and 3 – end of stage and INN – inoculum

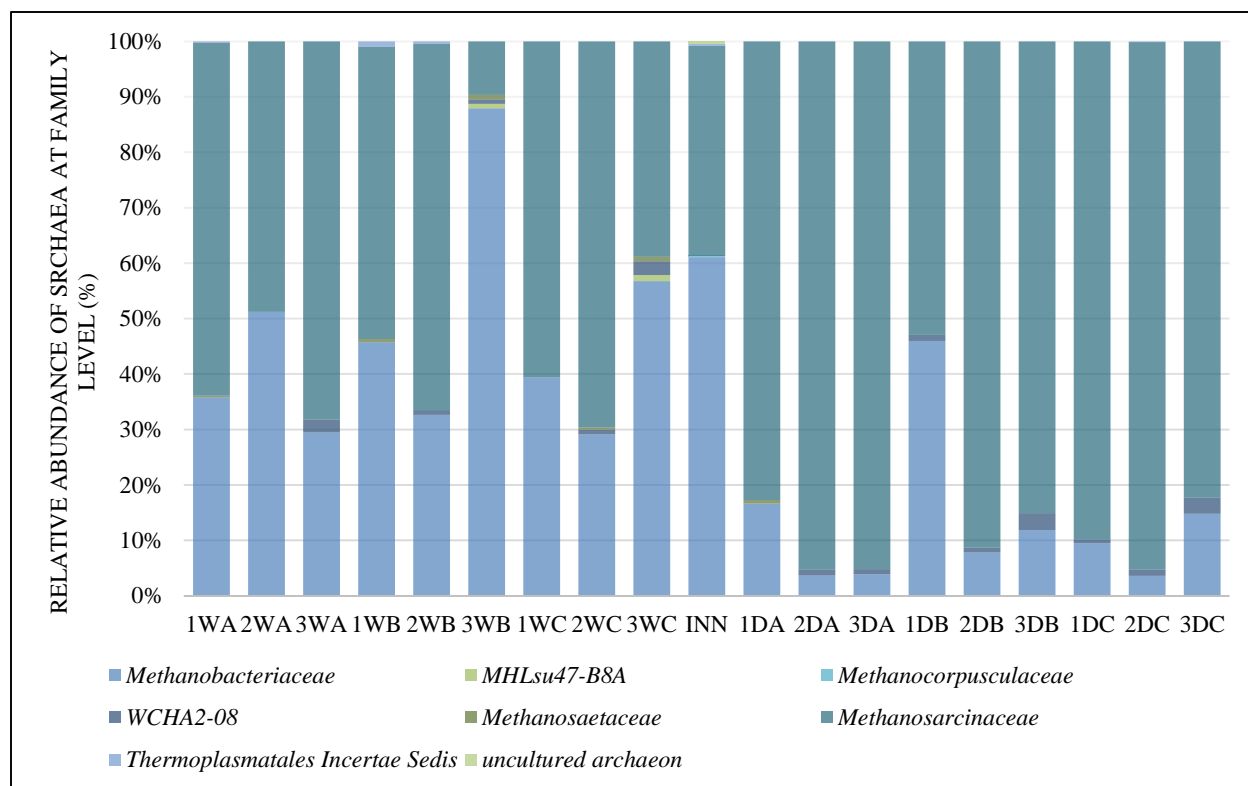


Figure 4.23: Relative abundance of archaea at family level for mono- (W) and co-digestion (D). A – stage 1, B – stage 2 and C – stage 3, 1 – beginning, 2 – mid and 3 – end of stage and INN – inoculum

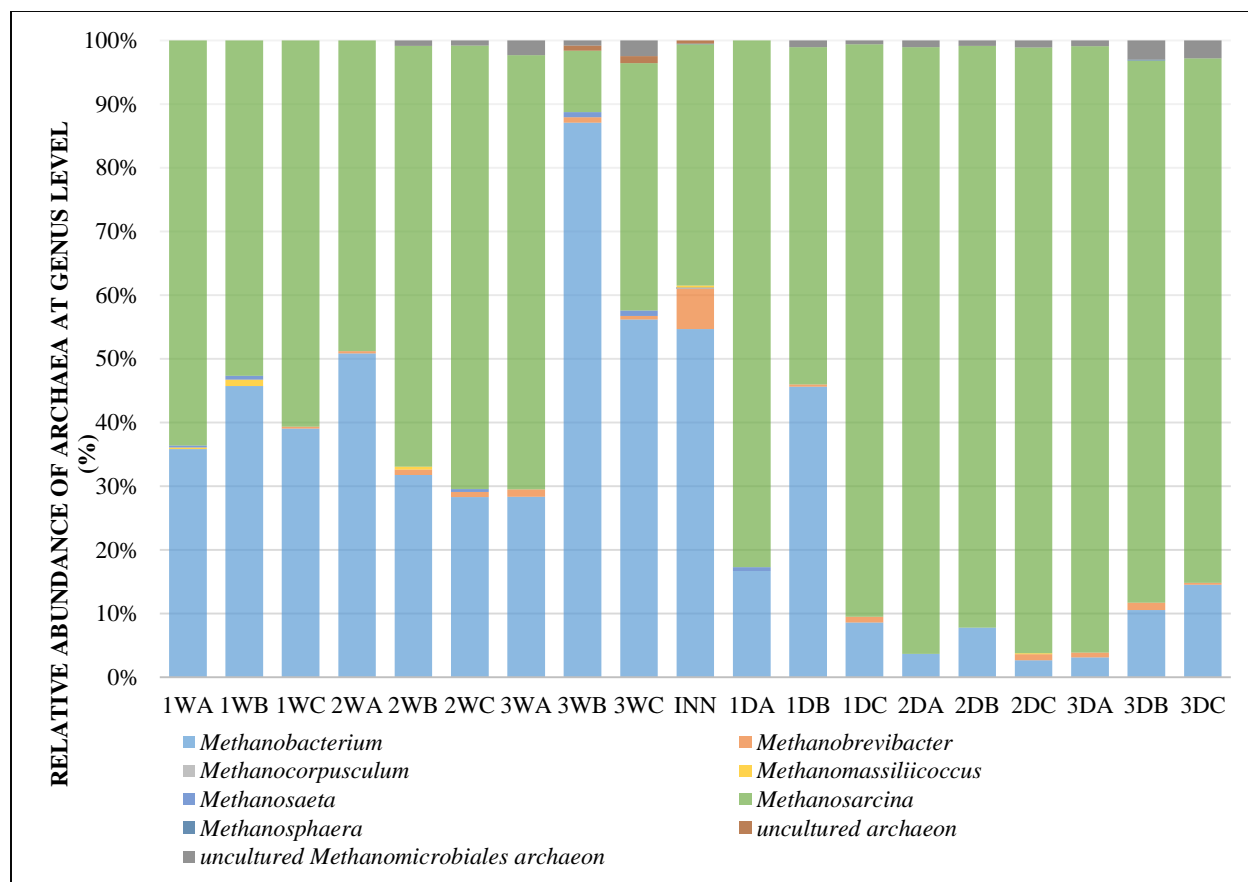


Figure 4.24: Relative abundance of archaea at genus level for mono- (W) and co digestion (D). A – stage 1, B – stage 2 and C – stage 3, while 1 –beginning, 2 – mid and 3 – end of stage and INN – inoculum

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1. Suitability of water hyacinth as feedstock for biogas production

The suitability of water hyacinth as a feedstock for biogas production was evaluated. The plant was found to be rich in carbon and nitrogen (Figure 4.1). Although 3% of nitrogen may appear low, in comparison with the other substrates such as animal manure (Ko et al., 2008), water hyacinth contains high nitrogen content (Gunnarsson & Petersen, 2007). Carbon is used as a source of energy while nitrogen is used for microbial growth by providing the essential elements used in the production of compounds such as proteins, nucleic acids and amino acids (Neubeck et al., 2016; Xie et al., 2012). In addition, nitrogen is converted to ammonia, which at low concentrations functions as a neutralising agent for maintaining the pH required for microbial cell growth whereas, at a higher concentration it may have toxic effects. The results obtained showed that water hyacinth contained a low C/N ratio which is associated with ammonia inhibition. The findings are in agreement with Gunnarsson and Petersen (2007), who showed water hyacinth to contain a low C/N ratio of approximately 15. It is widely accepted in the case of other substrates that an optimal C/N ratio of approximately 20-32 is required for CH₄ production without ammonia inhibition (Karthikeyan & Visvanathan, 2012; Li et al., 2011; Wang et al., 2014; Yen & Brune, 2007). However, the C/N ratio may not be the only significant parameter required for CH₄ production in substrates such as water hyacinth. This was evidenced by Jayaweera et al. (2007) where it was concluded that CH₄ production is not dependent on C/N ratio after comparing biogas production from water hyacinth with low C/N ratio, grown under different nitrogen concentrations.

Water hyacinth was also found to contain low amounts of potassium and phosphorus, similar results were obtained from Abdel-Sabour (2010) for phosphorus, whereas potassium was much lower than what was observed in this study (around 3.6%). These macronutrients are also known to be important during anaerobic digestion. For example, potassium is known to increase cell wall permeability and is used mainly by methanogens during AD (Wu et al., 2016a). In addition, the plant contained important micronutrients such as nickel, molybdenum, selenium and tungsten (Figure 4.2 A and B). This study found the presence of these micronutrients in higher percentages in the roots as compared to the leaves and the petioles. This was however, expected because roots are the main entry point of nutrients before they are transported to other parts of the plant. Similar results were obtained from Abdel-Sabour et al. (1996), where it was observed that in the case of heavy metals, the roots had higher concentrations in comparison to the shoots and leaves. Some

of these metals are essential and are found in the active sites of enzymes responsible for the conversion of complex compounds during hydrolysis (Neubeck et al., 2016). The outcome of the AD process is dependent on the activity of the microbial community, while the activity of microbial community is dependent on the availability of certain nutrients. Thus, the presence of these macro and micronutrients makes water hyacinth a suitable feedstock for AD.

5.2. Water hyacinth pre-treatment methods

5.2.1 Physical pre-treatment methods

CH₄ production from all the treatments (H, HC, OD and SD) and controls commenced within 3 days of fermentation (Figure 4.3) although the CH₄ produced in the treatments increased greatly in comparison to the controls. This was probably due to the presence of active microorganisms that had already adapted to the CH₄ production, obtained from the actively digested cow dung slurry (Xie et al., 2012). Because the microorganisms had adapted to the CH₄ production, their presence resulted in the highest CH₄ production on day 17 in comparison to day 21 from the controls. As for the controls, the multiplication and adaptation of microorganisms to the environment during the lag phase was probably lengthy and prolonged the production of the highest amount of CH₄ (Xie et al., 2012). In addition, the active microorganisms allowed the ongoing increase of CH₄ production from the treatments to last for 14 days as compared to the 4 days only observed for the controls before stabilisation, Chen and Hashimoto (1996) reported similar results. Low CO₂ production for both the treatments and controls was observed as the CH₄ increased. Similar results were obtained by Rotaru et al. (2014); Arthur et al. (2011) and Chanakya et al. (1993). This effect may be due to the complete uptake of CO₂ by hydrogenotrophic methanogens, which reduces CO₂ in the presence of hydrogen to produce CH₄ (Rotaru et al., 2014).

The differences observed in the pH value trend between the treatments and the controls emphasised the importance of co-digestion with actively digested cow dung slurry, which is known to possess qualities such as a buffering capacity and a variety of compounds (Kennedy et al., 2015). The pH for all the treatments increased after AD because of the presence of the microorganisms that were able to convert protein-rich organic matter into compounds such as carbonate and bicarbonate that neutralised the acid produced during the first three stages of AD (Kennedy et al., 2015). This process is known as alkalinity and is preferred during AD because it maintains the pH between 7 and 8. The low pH observed in the H control after AD proved that the variation in compounds and

microorganisms from the addition of inoculum assist in stabilising the pH. The H control lag phase lasted for about 29 days. This was possibly due to the low pH, which affected the activity of the methanogens.

5.2.2 A combination of physical and biological pre-treatment

For the physical and biological pre-treatment method, decomposition was carried out for 7 days by resident microorganisms. During the biological pre-treatment process, the microorganisms consume some of the carbohydrates, thus reducing the quantity of carbohydrates that remain after pre-treatment (Agbor et al., 2011). This was confirmed when the impact of pre-treatment on lignocellulosic composition was compared (Table 4.1). The HCD pre-treatment resulted in a lower recovery of cellulose and hemicellulose in comparison to the physical pre-treatment methods. In addition, for water hyacinth, 7 days of decomposition was too long. Other studies using the same substrate allowed 1 or 2 days for microbial decomposition (Ofoefule et al., 2009). It was also observed that the HCD pre-treatment method resulted in the recovery of the highest lignin content. The slow increase in CH₄ (Figures 4.5 A and B) production throughout the process for the HCD and HCD controls may be related to difficulties in the accessibility of nutrients by the microorganisms due to high lignin content, thus delaying hydrolysis step. Another factor could be the production of inhibitory compounds from the degradation of sugar and lignin that inhibit microbial growth and enzymatic activities (Agbor et al., 2011). This may be the reason why the function of the inoculum, as seen from the physical pre-treatment method, was not observed in this case. Although slight differences were observed between the HCD and HCD controls, the pH of the HCD and HCD control before and after AD remained within the optimal pH required for an effective AD process. This explains the notion that degraded sugars and fermentation inhibitors may have been the main reasons for HCD failing to enhance CH₄ production. Similar to the physical pre-treatment methods, low CO₂ for HCD was observed as the CH₄ increased.

5.2.3 Effect of pre-treatment on substrate biodegradation

The purpose of pre-treatment methods is to make organic matter available for enzymatic degradation thus, increasing biodegradation rates. The physical pre-treatment mainly focused on varying the particle size while a combination of physical and biological pre-treatment focused on size reduction as well as the use of naturally occurring aerobic microorganisms to expose the biopolymers. Theoretically, high availability of organic matter is associated with high

biodegradation rates (Lesteur et al., 2010). The slight differences in CH₄ production observed among the treatments at day 17 and 35 (Figure 4.6) was due to the ability of the individual pre-treatment methods to make organic matter accessible and the biodegradation extent of the available organic matter (Raposo et al., 2012). In addition, CH₄ production depends on the chemical composition of the pre-treated substrate (Rubia et al., 2011).

The differences in the order of highest to lowest between the cumulative CH₄ production (Figure 4.6), the percentage reduction of VS after AD (Table 4.3) and the CH₄ yield (Table 4.4) may be explained by the different chemical composition of the substrates after their exposure to various pre-treatment methods. Although the same substrate was used, the pre-treatment methods resulted in variations in the available chemical composition within the substrate. This is possible because the differences in chemical composition are not limited only to the different plant species but may also be observed within the same plant species (Mészáros et al., 2004). This was also observed in a study conducted by Rubia et al. (2011). They evaluated the influence of different particle sizes obtained from pre-treatment methods on CH₄ production and concluded that the various segments of the particle sizes contained different chemical composition.

The method used to calculate the LCH₄ produced per VS was based on a theory that the chemical compositions within VS are the same (Frigon & Guiot, 2010). However, not all VS are equal as some contain poorly degraded compounds such as lignin (Frigon & Guiot, 2010), while others contain more carbohydrates than proteins or lipids. The degradation of proteins or lipids produces more CH₄ than the degradation of carbohydrates (Angelidaki & Sanders, 2004). In this study, similar amounts of VS were added at the beginning of the experiment for all the treatments. This explained the similar CH₄ yield on day 35 in all the physical pre-treatments. This finding is in agreement with the results of the study conducted by Moorhead and Nordstedt (1993). The difference in the final CH₄ content may possibly be attributed to the difference in the chemical composition based on the pre-treatment method used.

5.2.4 Selection of optimal pre-treatment method

The ultimate purpose of evaluating the different water hyacinth pre-treatment methods was to select the most efficient method for maximal CH₄ production. All the physical pre-treatment methods significantly enhanced CH₄ production in comparison to the HCD. When compared to each other, the samples from the four physical pre-treatment methods tested did not differ

statistically in their CH₄ production. However, a single physical method had to be selected for future trials.

Although OD performed well, the pre-treatment method was not selected as the best method. Oven dried water hyacinth had the smallest particle size, thus implying that it had an increased surface area to volume ratio. This was confirmed by the amount of VS reduced after AD (9.87%). However, as observed in Figure 4.6, the OD had the same LCH₄ as H and HC, which caused a reduction of 7.1 and 5.09% of VS respectively after AD. Theoretically, the highest VS reduction corresponds to the highest CH₄ yield but, in practice, the CH₄ yield depends on the type of chemical compounds available. The process of oven drying and grinding resulted in small particles which are associated with the availability of easily degraded compounds such as carbohydrates. The CH₄ production potential of carbohydrates is lower than that of proteins and lipids.

Angelidaki and Sanders (2004) showed that different particle sizes vary in the amount of carbohydrates, proteins and fats, with the smallest particle known to contain high amounts of carbohydrates. Studies from Izumi et al. (2010); Moorhead and Nordstedt (1993); Raposo et al. (2012) and Rubia et al. (2011) evaluated the CH₄ production from different particle sizes and found the smallest particle produced the lowest amount of CH₄. The low CH₄ production was due to the presence of high amounts of easily degraded carbohydrates. However, Izumi et al. (2010) suggested a different reason, explaining that the lower CH₄ production from the smallest particle was due to accelerated hydrolysis and acidogenesis. This leads to the production of high concentrations of VFAs which affect the activities of the methanogens. In terms of economic feasibility, the process of oven drying and grinding requires high energy input and is time-consuming. Furthermore, the process of drying removes water from the plant, thus implying that the addition of more water would be necessary during AD in comparison to non-dried feedstock.

Homogenised pre-treatment method was the second method that showed high substrate biodegradability (Table 4.3). However, the method was not selected as the best method. Homogenised control was the only control method that resulted in process failure, thus implying that the process of homogenising destroyed or damaged the cell membrane of most of the methanogens in the plant and that the majority of them were unable to recover during AD. It is known from the literature that methanogens grow at a slower rate than acid producing anaerobic bacteria (Chen et al., 2016). The acidic pH observed at the end of the process indicated that the

activity of the methanogens was minimal which resulted in the accumulation of VFAs. The H pre-treatment with inoculum performed well due to the presence of active microorganisms from the inoculum. However, if the activity of the inoculum is compromised, a process failure may well be expected if this pre-treatment method is used.

Sun-dried showed substrate biodegradability of 6.09% with a slightly different cumulative LCH₄ production as compared to HC, OD and H. However, this pre-treatment method was not selected as the best method. It is clear from Figure 4.6 that, from day 3 to day 17, SD showed low degradation rates. However, after day 17 the degradation rates increased which resulted in the highest CH₄ production on day 29. Thus, this pre-treatment method failed to increase the biodegradation rates at the beginning of the process. It must be remembered that the purpose of pre-treatment is to increase the rate of hydrolysis and to obtain the highest CH₄ yield faster than would otherwise have been possible. In addition, the process of sun drying is time-consuming and, like the OD pre-treatment method, it reduces water content.

Hand cut was the pre-treatment method which was selected as the best pre-treatment for water hyacinth. It was observed that HC showed low substrate biodegradability rates although it was able to produce a CH₄ yield similar to OD, H and SD, which had higher percentages of reduced VS. This showed that, in comparison to OD, H and SD, this type of pre-treatment method resulted in the bioavailability of more proteins and lipids with a higher CH₄ potential than that of carbohydrates, thus resulting in higher CH₄ production using less VS. Scissors were used for cutting the plant due to the small amount of sample tested. However, for upscaling, a shredder that can reduce the water hyacinth particle size from 2.5 to 2 cm could be used to ensure the feasibility of this pre-treatment method. Advantages HC pre-treatments include reduced time for pre-treatment as well as the simplicity of the process (no heating).

5.3. Effects of irregular OLR on process stability, performance and microbial community

This section of the study investigated the effects of irregular OLRs on the AD of water hyacinth (mono- and co-digestion) in biogas production. The effects of irregular OLR on bacterial and archaeal community composition structure were also evaluated using a 16S rRNA gene-based metagenomics approach.

5.3.1 Process stability and performance

The process stability of the two digesters in this study was evaluated by monitoring the pH and FOS/TAC ratio (Kennedy et al., 2015; Scano et al., 2014). The pH of the substrate in a digester reflects the approximate state of the digester but cannot be used as an early indicator of process stability (Kennedy et al., 2015). The pH obtained in both mono- and co-digestion remained within the acceptable range for AD but not within an optimum range of between 6.8 and 7.4 (Schloss et al., 2009). In co-digestion, the pH was slightly higher than in mono-digestion, indicating the buffering capacity of the cow dung. A study by Yi et al. (2014), measured the pH and VFAs as the TS were increased. Their results showed an acceptable pH range in one of their digesters with a high concentration of VFAs being observed, thus implying the inaccuracy of pH as an appropriate early indicator of process stability in a substrate with good buffering capacity. The pH of the mono-digestion was also within an acceptable range, thereby emphasising the suitability of the plant in mono-digestion.

Irregular OLRs are known to cause a decrease in biogas production. The difference in biogas production was observed between mono- and co-digestion when irregular OLRs were introduced. The continuous increase in biogas production in mono-digestion may be correlated with the optimal FOS/TAC ratio within a range of 0.4 to 0.6 (Figure 4.12) which is indicative of process stability (Rincón et al., 2008) especially during stages 1 and 2, although optimum biogas production with high CH₄ yield was observed in stage 2. Maximum biogas production with a high CH₄ yield (Figure 4.11) was observed when the FOS/TAC was in a range of 0.4 to 0.6, thus implying that, for water hyacinth, this range can be linked to stable operating conditions. Similar to the study conducted by Wan and Li (2011), the ratio of between 0.4 and 0.5 was obtained during the stable operation period for activated sludge waste. However, a study by Allen et al. (2014), in which they co-digested seaweed and slurry, observed an optimal FOS/TAC ratio of between 0.2 and 0.4 during stable operating conditions. In another study by Di Maria et al. (2014) a ratio of < 0.1 was observed during stable conditions for fruit and vegetable waste digestion. According to Scano et al. (2014), the FOS/TAC ratio is influenced by the type of substrate used.

The high FOS/TAC ratio in mono-digestion stage 3 was indicative of excessive biomass input (>0.6), implying that the process was overloaded, thus resulting in a decrease in CH₄ yield due to loading shock (Figure 4.12). Studies by Di Maria et al. (2014) and Scano et al. (2014) showed that

increasing feedstock or OLRs increases the FOS/TAC ratio and it is, therefore, recommended that feeding is reduced for the FOS/TAC ratio to decrease. Accordingly, in stage 3 of mono-digestion, the high FOS/TAC ratio was due to increased TS (Scano et al., 2014). In co-digestion, the decrease in biogas production in stage 2 and process failure in stage 3 was also correlated with the FOS/TAC ratio, which was, in the main, below the recommended level. According to Lossie and Pütz (2008), a low FOS/TAC ratio implies that the digester is “hungry” and an increased biomass input is required. Although biomass input was increased in stage 2 and 3, the FOS/TAC ratio did not improve, so as the biogas production. This may be due to process instability, which was reflected by the low FOS/TAC ratio, thereby emphasising the importance of FOS/TAC ratio as an early indicator of process instability. In addition, stage 1 of co-digestion had a slightly higher FOS/TAC ratio as well as increasing biogas production, in comparison to two stage 2 and 3. Thus putting more emphasis that stage 2 and 3 of co-digestion were unstable, thus the low biogas production.

Overall, the changes imposed by irregular OLRs affected both the amount and quality of the biogas produced. In mono-digestion, stages 2 and 3 resulted in increased total biogas production although the total CH₄ production decreased (Table 4.5). In addition, the percentage of CH₄ in the biogas produced decreased in stages 2 and 3, thus resulting in slightly higher percentages of CO₂.

5.3.2 Bacterial community structure and composition

In addition to affecting the biogas production, the irregular OLRs also affected the microbial community structure and composition (Regueiro et al., 2014). In mono-digestion, the genus *Bacteroides* was dominant at the beginning of each stage and decreased as the stages progressed. The genus *Bacteroides* is known for its ability to biodegrade complex plant polysaccharides, such as cellulose (Hatamoto et al., 2014; Shah & Williams, 1987), and its high abundance at the beginning of mono-digestion which relates to the ability to consume cellulose from water hyacinth. According to Shah and Williams (1987), the capabilities of the genus are linked to the nutrients available in the environment, thus implying that the nutrients in mono-digestion favoured *Bacteroides* growth. In addition, this happens during hydrolysis. The decrease in the dominance of *Bacteroides* at the mid and end sampling points showed that the cellulose content had been converted to other compounds with this being the reason why, in the mid and end sampling periods of the stages other groups, such as *Proteiniphilum*, *Acinetobacter*, *Christensenellaceae* R 7 group, *Petrimonas* and *Paludibacter*, increased.

The genus *Proteiniphilum* is a proteolytic bacterium, while *Paludibacter* is a saccharolytic bacterium (Chen & Dong, 2005; Ueki et al., 2006). In addition, according to Ziganshin et al., 2011, these genera are known to produce elevated levels of both acetate and propionate. Acetate is known as a product that is produced during acetogenesis (a precursor for methanogenesis) (Yadvika et al., 2004) and this explains their dominance at the mid and end sampling points of mono-digestion. On the other hand, acetogenesis is carried out by the metabolic gene formyltetrahydrofolate synthetase (FTHFS) which codes for a key enzyme in reductive acetogenesis. All the samples from the mono-digestion contained this metabolic gene (Figure 4.14 A), thus indicating that acetogenesis had occurred in all the samples. Overall, the comparison of the microbial community structure and composition in all the sampling periods in all the stages showed variation – some bacterial genera decreased while others increased. OLRs affected both the dominance and the abundance of genera in each stage. This finding was supported by the alpha diversity indices, which fluctuated between the stages (Table 4.6).

In co-digestion, the beginning of each stage was dominated by different genera, although genera such as *Petrimonas*, *Bacteroides*, *Proteiniclasticum* were common. The difference in the dominant genera was influenced by the OLRs (Hansen et al., 1998; Karakashev et al., 2006; Regueiro et al., 2014; Tham, 2012; Zou et al., 2014) which favoured certain communities. In comparison to mono-digestion, the bacterial community composition in co-digestion showed a greater variation and were more abundant in each stage. This finding was also supported by the alpha diversity, which showed co-digestion to demonstrate higher bacterial diversity in comparison to mono-digestion. Figure 4.17 illustrates the variation between the genus *Bacteroides* and *Petrimonas* in both digestions. Ziganshina et al. (2015) mentioned that the diversity of the Bacteroidetes, Proteobacteria, Firmicutes and Chloroflexi phyla was influenced mainly by the substrate type and the OLRs. Accordingly, the variations in genera were probably influenced by the available nutrients in the substrate. In addition, the dominance of the phyla Bacteroidetes in this study may be related to organic overload because Bacteroidetes have been reported to be resistant to elevated levels of VFAs (Regueiro et al., 2014). Organic overloading is directly related to increases in VFAs.

In co-digestion, the mixture of cow dung and water hyacinth resulted in a low dominance of *Bacteroides*. Furthermore, the cow dung contained components that were already or partially degraded and the mixing of the two substrates resulted in a lower cellulose component as compared

to mono-digestion. The *Christensenellaceae* R 7 group is known to carry out both hydrolysis and acetogenesis (Wu et al., 2016b) and it was also observed that this genus increased in the samples when *Bacteroides* decreased. In co-digestion hydrolysis may, therefore, have been carried out by both *Bacteroides* and the *Christensenellaceae* R 7 group. An increase in *Petrimonas* in most of the samples at the beginning was observed. *Petrimonas* is a bacterium that ferments sugar to generate acetate although it may also use nitrate or elemental sulphur as electron acceptors (Grabowski et al., 2005; Nakasaki et al., 2009). The increase in *Petrimonas* from the beginning of the stage implied that acidogenesis and acetogenesis had occurred earlier in co-digestion and more rapidly than in mono-digestion. In Figure 4.14, the metabolic gene for acetogenesis, FTFHS, was not amplified for the two samples of co-digestion collected at the end and mid time points of stages 1 and 2 respectively. The reason for this is that when acetogenesis occurs rapidly, it produces VFAs, which, if not converted to CH₄ by methanogens, may result in the inhibition of acetogenesis (Cirne et al., 2007; Ziganshin et al., 2011).

Unique to stage 2 co-digestion, the genus *Pseudomonas* was found to be dominant at the mid and end sampling periods of stage 2. *Pseudomonas* is a facultative bacterium, and it is associated with the utilisation of oxygen accidentally introduced during feeding to create favourable conditions for obligate anaerobes (Hernon et al., 2006; Hernandez et al., 1991). Once the oxygen is depleted, the bacterium uses the denitrification process for respiration only if nitrate is available in the digester (Hernandez et al., 1991). The water hyacinth plants used in this study were harvested from a dam that was already in the state of hypertrophication, mainly from the nitrates and phosphate from agricultural and mining activities in the vicinity of the dam (Harding et al., 2004). The phytoremediation ability of the plant explains the possibility of the presence of nitrate in the digesters. In addition, the process of denitrification to produce nitrite from nitrate may be linked to the dominance of *Petrimonas* in co-digestion, which is known to use nitrate as an electron acceptor to produce ammonia. *Acinetobacter* was found to be dominant in both mono- and co-digestion although more dominant in most of the samples of co-digestion, this may have contributed to the low biogas production in co-digestion. According to Chen et al. (2017) and Su et al. (2015) both *Acinetobacter* and *Pseudomonas* are capable of carrying out denitrification and nitrification – the conversion of nitrate and ammonia into nitrogen gas.

5.3.3 Archaeal community structure and composition linked to Bacterial community and analytical methods

The importance and success of AD is attributed primarily to the activity of the archaeal community, the producers of CH₄ and other gases. The type of archaeal community present in the digester is dependent on the type of precursors produced during acetogenesis, as well as whether the process is overloaded or not. In mono-digestion, *Methanosarcina* and *Methanobacterium* dominated all the stages although *Methanosarcina* was slightly higher in some of the samples. *Methanobacterium* is a hydrogenotrophic methanogen while *Methanosarcina* is an acetoclastic methanogen, although *Methanosarcina* is known to use both the hydrogenotrophic and acetotrophic pathways in CH₄ production. The amplification of the *mcrA* gene in all the samples of mono-digestion (Figure 4.14 B) showed that methanogenesis was conducted mainly via the hydrogenotrophic pathway. This also implied that the conditions in the digesters favoured the hydrogenotrophic pathway in the CH₄ production by *Methanosarcina* rather than the acetotlastic pathway. *Methanobacterium* and *Methanosarcina* are known to be resistant to elevated levels of VFAs (Franke-Whittle et al., 2014) while *Methanosarcina*'s growth increases when elevated levels of VFAs (especially acetate) are detected in the digester (Demirel & Scherer, 2008). The presence of both genera, especially *Methanosarcina*, in mono-digestion could be related to organic overloading.

The dominance of the genus *Methanosarchina* has been reported in cases in which the AD process is regarded as overloaded (high levels of VFAs) (Schloss et al., 2009). Furthermore, *Methanosarcina* genus is known to have a high growth rate in comparison to other methanogens and it is able to tolerate changes in pH as well as a high concentration of toxic compounds (Demirel & Scherer, 2008).

The dominance of *Methanosarcina* throughout stages 2 and 3 in co-digestion also further explain that bacteria are more sensitive to OLRs than archaea. In order to elaborate on this further, both *Methanosarcina* and *Methanobacterium* dominated the beginning of stages 1 and 2. As the stages progressed *Methanobacterium* drastically reduced leaving *Methanosarcina* as the dominant methanogen. This was also shown by the decrease in the alpha diversity for stages 2 and 3 (Table 4.7). The decrease in the dominance of *Methanobacterium*, which produces CH₄ via hydrogenotrophic pathway, may be explained by the presence of more acetate-producing bacteria,

which favour the dominance of *Methanosarcina*. This was evidenced in Figure 4.14C, where the metabolic gene for producing CH₄ via acetoclastic pathway was amplified mostly in co-digestion samples. In addition, the metabolic gene *mcrA* was amplified in all samples of co-digestion, further explaining the dominance *Methanosarcina* due to its ability to produce CH₄ using the two pathways. Stages 2 and 3 had higher VS content in comparison to stage 1. It is known that increasing TS will result in higher concentrations of VFAs, especially acetate, which in turn favours the growth of *Methanosarcina* that used acetate to produce CH₄ in stages 2 and 3 (de Vrieze et al., 2012). In addition, high VFAs cause a decrease in the FOS/TAC ratio, as was observed during stages 2 and 3 of co-digestion, thus indicating the need to add biomass due to the rapid biodegradation of the biomass.

As mentioned above, *Methanosarcina* is found mainly in digesters with elevated levels of VFAs while the high prevalence of *Methanosarcina* in such a digester would still lead to CH₄ production (Yi et al., 2014). In co-digestion, the biogas production continuously decreased until process failure was observed in stage 3. Co-digestion was dominated by *Methanosarcina*. This may have been due to the presence of certain bacterial communities and their products that created environmental variations in the digester which had an effect on the activity of *Methanosarcina*. The presence of *Acinetobacter* and *Pseudomonas* may have contributed to the continuous decrease in biogas production during co-digestion (Clarens et al., 1998; Chen et al., 2017; Su et al., 2015). Their presence signalled the removal of nitrate from the digesters and, during the denitrification of nitrate to nitrogen gas, the intermediate nitrite was produced. According to Clarens et al. (1998), the presence of nitrite (approximately 0.18 mM) shows a higher inhibitory ability to methanogenesis than may otherwise have been the case. Furthermore, the dominance of *Petrimonas* in co-digestion also confirmed the presence of nitrite and its conversion to ammonia.

Both the increase in and dominance of *Petrimonas* were related to the availability of nitrate. Thus, in co-digestion, the nitrate removal was elevated and the denitrifying bacteria may have turned to CH₄ as an electron donor for the removal of nitrate (Costa et al., 2000), thus explaining the continuous decrease in CH₄ production in co-digestion. In addition, under limited oxygen conditions, CH₄ is used as an electron donor during denitrification (Costa et al., 2000; Islas-Lima et al., 2004; Raghoebarsing et al., 2006; Westermann & Ahring, 1987). In mono-digestion, *Acinetobacter* was dominant either at the beginning, mid or end points of the stages whereas, in co-digestion, *Acinetobacter* was dominant in all the samples (except the end sampling point of

stage 3), thus implying that co-digestion environment was more conducive for *Acinetobacter* and *Pseudomonas* growth as compared to mono-digestion.

5.3.4 Comparison of diversity for both digesters.

Different results were obtained statistically from PERMANOVA and ANOSIM. This was, however, expected because both the PERMANOVA and ANOSIM do not analyse the same properties of the data. PERMANOVA tests the differences (similarities) between groups while ANOSIM tests whether the distribution is unique or not. In addition, different statistical results were obtained for both the weighted and unweighted beta diversity measures. For the weighted both PERMANOVA and ANOSIM showed no significant difference (supported by the low F-pseudo and R value for both). The fact that weighted takes into account the relative abundance of bacteria clearly explains that the difference in bacterial abundance was not significant. For the unweighted, which takes into account the presence or absence of species, PERMANOVA was significantly different while ANOSIM was not. This, in turn, provided an explanation for the finding that the diversity was different (based on presence or absence), thus supporting the notion that the substrate composition (whether mono- or co-digestion) contributed to microbial community selection. ANOSIM showed that the distribution was not unique. For weighted archaeal PCoA, similar results were obtained, also indicating no difference in diversity. However, for unweighted archaeal PCoA both PERMANOVA and ANOSIM were significantly different. This may be related to the difference in the dominance and abundance of *Methanobacterium* and *Methanosarcina* in both the digesters.

5.4. CONCLUSION

The problems caused by the infestation of water hyacinth in water bodies as well as the ongoing failure of the available control methods have resulted in the need for alternative control methods that are environmentally friendly. The anaerobic digestion of water hyacinth for the production of biogas rich in CH_4 has been identified as a potential alternative control method for water hyacinth infestation. Accordingly, this study focused on identifying the biotechnological processes that could improve the suitability of water hyacinth as feedstock for biogas production, these included the pre-treatment of water hyacinth to improve methane yield as well as the microbial community involved in anaerobic digestion of water hyacinth.

The pre-treatment objective was a screening stage, in which different pre-treatment methods were tested with the purpose of selecting the best suitable method for water hyacinth. The results revealed that all the physical pre-treatment methods enhanced methane production. Methane production is dependent on the quantity of the biodegradable organic matter present in the substrate during AD. The process of pre-treatment is responsible for increasing the biodegradability of the substrate by exposing the biopolymer to microorganisms and eventually enhancing their conversion to methane during the AD process. This study revealed that simple pre-treatment methods, such as size reduction, are suitable for exposing the biopolymers of water hyacinth to biodegradation during AD. The CH_4 production during batch assays for both the treatments and the controls demonstrated the suitability of the plant as feedstock for biogas production.

The performances of microbial communities in AD process are dependent on the available nutrients and the results from the chemical analysis of water hyacinth showed that water hyacinth contains essential macro and micronutrients at a required range to support microbial growth and activity during anaerobic digestion. Although the plant had low C/N ratio in comparison to other substrate used for biogas production, this did not affect the CH_4 production in both batch and semi-continuous AD.

The optimal pre-treatment method selected as the best method for water hyacinth did improve biogas production in semi-continuous AD. However, once irregular OLR was initiated, changes in biogas production was observed in both mono- and co-digestion, with co-digestion leading to process failure. The analysis of microbial community structure suggested that the irregularity in OLRs had an effect on the microbial community structure and, ultimately, the quality of biogas

produced. Although the effects of irregular OLRs on digester stability, performance and the microbial community were observed in both mono- and co-digestion, this was more prevalent in co-digestion. The comparison of the abundance and dominance of the bacterial community between mono- and co-digestion showed variations. Substrate composition played a role in the bacterial diversity in both digestions. In addition, it was observed that bacterial communities were more sensitive to OLRs in comparison to the archaeal community. In this study, the abundance of the archaeal community, such as *Methanosarcina* and *Methanobacterium*, was not influenced by the changes in OLRs but, rather, by the type of products produced during the previous stages, thus indicating the importance of the bacterial community in the AD processes.

The dominance of genera such as *Bacteroides*, *Petrimonas*, *Acinetobacter* and *Pseudomonas* is an example that shows that the type of substrate, mainly the presence of nutrients, contributes to the selection of microbial communities. Co-digestion is usually associated with variations in nutrients as compared to mono-digestion. The results from this study showed high numbers of different active bacterial communities in co-digestion as a result of nutrient variations as compared to mono-digestion. However, in this study, this led to process instability in co-digestion due to the rapid degradation of organic matter.

Overall, biogas production from water hyacinth as an alternative control method for water hyacinth infestation is a possibility. The study revealed that water hyacinth has the ability to produce CH₄ when anaerobically digested in the absence of an inoculum, as observed from batch assays controls. Furthermore, digesting water hyacinth as a mono-substrate provided evidence of the ability of the microbial community composition to withstand OLR disturbances without process failure as was observed in semi-continuous AD. This suggests the potential use of water hyacinth as a single substrate for biogas production.

5.5. RECOMMENDATIONS

Anaerobic digestion studies focus primarily on methanogenesis that is driven by the archaeal community. However, the bacterial community also plays a key role and, without the activity of the bacterial community, methanogens would not have the precursors to use in methanogenesis. As shown in the study results, the presence of certain bacterial species may be related to process efficiency or inefficiency. Thus, a greater understanding of the bacterial community during AD is recommended especially in relation to disturbances by OLRs and type of substrate. Such

understanding will be important for the selection of bacteria that may be used as indicators of the type of methanogenic communities and possible inhibitors expected.

In addition, the floating characteristic of water hyacinth on the surface of water as well as the design of the digesters created a number of the challenges encountered during semi-continuous AD.

- During semi-continuous feeding, the water hyacinth floated on the top of the digester (Appendix A5.1 B). The floating of the substrate may have resulted in the slow biodegradation of the substrate initially. The stirring speed used in the study had little effect on the first day of feeding, however, as the plant material is digested/biodegraded it becomes mixed in the solution.
- Another challenge was the design of the two digesters used in the study. The feeding pipe was situated close to the edge of the digesters (Appendix A5.1 A). This resulted in the water hyacinth being stuck in the space between the feeding pipe and the digester edge (Appendix A5.1 C). The stirring speed also had little effect on the substrate stuck in the sides of the digester. A high speed was not ideal for the experiment as it would have caused disturbances and, ultimately, affected the microbial community adherence to the substrate. Although the substrate was stuck, biogas production was still observed, as seen in Appendix A5.1 C, which showed bubbles between the samples. This is indicative of diffusing biogas.

The lab-scale, semi-continuous anaerobic digesters used in this study were designed to accommodate different types of substrates. It was clear from the challenges encountered in the study that the digester designs were the main reason for most of the challenges encountered. Thus, it is recommended that anaerobic digesters for substrates such as water hyacinth, be built specifically to best suit water hyacinth and, perhaps, other types of macrophytes.

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ADDENDUMS

Appendix A3.1: Permit for conduction research on water hyacinth


REPUBLIC OF SOUTH AFRICA

Department of Environmental Affairs
Issuing Authority, Department of Environmental Affairs, Directorate: Biosecurity Services, Private Bag X 4390, Cape Town, 8000
14 Loop Street, Cape Town, 8001. Tel: 021 441 2748, E-mail: AlSPermits@environment.gov.za

**THIS PERMIT IS ISSUED IN TERMS OF CHAPTER 7 OF THE NATIONAL ENVIRONMENTAL
MANAGEMENT: BIODIVERSITY ACT, 2004 (ACT 10 OF 2004) (AS AMENDED) READ TOGETHER WITH
THE ALIEN AND INVASIVE SPECIES REGULATIONS, 2014 (AS AMENDED)**

PERMIT FOR RESTRICTED ACTIVITY OF ALIEN SPECIES OR LISTED INVASIVE SPECIES

Permit Holder Details:

Name: Rasheed Adegbola Adeleke
Address: 600 Belvedere Street, Arcadia, Pretoria, 0083

I.D/ Company Registration number: 7604026368185
Permit number : 5086577918
Date issued: 29/08/2017
Expiry date: 31/12/2018
Amount Paid: R100.00

Exporter/Supplier/Seller/Trader details:

Name: N/A
Address: N/A


Premises where restricted activity will be carried out:
Physical Address: 600 Belvedere Street, Arcadia, Pretoria, Gauteng, 0083

Permission is hereby granted to the Permit Holder for the following: Possession/ Research under the attached permit conditions.

Species details

Scientific Name	Common Name	Quantity	Note
<i>Eichhornia crassipes</i>	Water hyacinth	± 1000	Plant species

Permit issued by: Khathutshelo Nelukalo
Place: Cape Town

Signature: 

Official stamp

DEPARTMENT OF ENVIRONMENTAL AFFAIRS
REPUBLIC OF SOUTH AFRICA
DIRECTORATE: BIO-SECURITY
29 AUG 2017
PRIVATE BAG X4390
CAPE TOWN 8000
ALIEN AND LISTED INVASIVE SPECIES: PERMIT

000002037

PERMIT NUMBER: 5086577918



environmental affairs

Department:
Environmental Affairs
REPUBLIC OF SOUTH AFRICA

Issuing Authority: Department of Environmental Affairs · Directorate: Biosecurity · Private Bag X 4390 · Cape Town · 8000 · 14 Loop Street · Cape Town 8001 · Tel: 021 441 2748 · Email: AISpermits@environment.gov.za

GENERAL PERMIT CONDITIONS FOR RESEARCH PURPOSES INVOLVING ALIEN AND INVASIVE SPECIES IN TERMS OF THE REGULATIONS OF THE NATIONAL ENVIRONMENTAL MANAGEMENT: BIODIVERSITY ACT, 2004 (ACT NO.10 OF 2004).

1. This permit shall not absolve the permit holder from complying with any obligations that he/she may have in terms of any other law.
2. The permit holder is referred to Regulations 18 – 29 of the Alien and Invasive Species (AIS) Regulations, the provisions of which are applicable to this permit, irrespective of whether they are specifically set out herein or not.
3. This permit is not transferable and relates only to the person / institute to whom it is issued.
4. This permit is valid only:
 - a) In its original form as issued by the Issuing Authority and any unauthorised alteration thereof and/or tampering therewith shall render it invalid unless subjected to condition 11 below;
 - b) For the period specified in the permit unless it is cancelled, revoked or amended by the Issuing Authority.
5. This permit shall automatically lapse if the species to which it relates is subsequently listed as a prohibited invasive alien species in terms of Section 67(1) of the Act.
6. The permit holder must prevent the spread of the permitted species and must control any specimen that spreads.
7. The permit holder shall at all times have in his/her possession this permit and permit conditions while performing any restricted activity and shall be produced when requested by any authorised official(s).
8. The permit holder will be subject to the provisions of Chapter 7 of the National Environmental Management Act 107 of 1998 in respect of the mandate and powers of Environmental Management Inspectors. The permit must be made available upon request by any authorised official designated by the Minister of Environmental Affairs. The permit holder shall allow the Issuing Authority or any other authorised official(s) unrestricted access to monitor compliance with permit conditions and for any inspection.
9. The permit holder must comply with any conditions or control measures of the approved available Risk Assessment documentation and/or submitted with the application for a permit.
10. Any incidents related to the permitted activities that are actual or potential threats to the environment and/or the breach of a condition of this permit must be reported in writing to the relevant Authority or his/her designated representative within 24 hours of the incident.
11. The Issuing Authority may amend, cancel or withdraw these permit conditions in writing after consultation with the permit holder.



PERMIT NUMBER: 5086577918



environmental affairs

Department:
Environmental Affairs
REPUBLIC OF SOUTH AFRICA

Issuing Authority - Department of Environmental Affairs - Directorate: Biosecurity - Private Bag X 4390 - Cape Town - 8000 - 14 Loop Street - Cape Town 8001 - Tel: 021 441 2748 - Email: AISpermits@environment.gov.za

12. The specimens must be used solely for the purpose stated in the permit and may not be used for any other purpose.
13. The permit holder must immediately notify the Issuing Authority of any change of address, premises and/or legal status in writing.
14. A permit that has been cancelled in terms of Section 93 of the Act must be returned to the Issuing Authority within 30 calendar days of the date of cancellation.
15. The permit holder hereby agrees to indemnify and hold harmless the Department of Environmental Affairs ("the Department"), including its authorised representatives, and/or a Provincial Department, including any authorised representatives, for any loss and/or damage, which may be incurred or suffered by the permit holder as a result of the Department, Provincial Department, and any of its authorised officials, reasonably and lawfully carrying out any of its responsibilities and/or duties and/or functions under any relevant legislation.
16. The permit holder may apply for the renewal of a permit 60 days before the expiry of the period for which the permit was issued.

CONDITIONS FOR POSSESSION OF *EICHHORNIA CRASSIPES* FOR RESEARCH PURPOSES.

17. This permit is only valid for the species and location indicated on the permit.
18. The plant material of *Eichhornia crassipes* sample(s) shall only be handled in facilities at The Agricultural Research Council (ARC) Institute for Soil, Climate and Water.
19. The plant materials shall only be used for research purposes only.
20. The sample(s) in a sealed container(s) shall be addressed to: Prof. Rasheed Adegbola Adeleke (+27 (0)728843243 or +27 (0)123102519)
21. Name of institution / Company: The Agricultural Research Council (ARC) Institute for Soil, Climate and Water
Postal address: Private Bag X79, Pretoria, 0001
22. The container shall be opened and the material handled in the approved facilities at:
Institution: The Agricultural Research Council (ARC) Institute for Soil, Climate and Water
Physical address: 600 Belvedere Street, Arcadia, Pretoria, 0083
Town/City: Pretoria, Gauteng, 0083
Name of responsible person: Prof. Rasheed Adegbola Adeleke



PERMIT NUMBER: 5086577918



environmental affairs

Department:
Environmental Affairs
REPUBLIC OF SOUTH AFRICA

Issuing Authority: Department of Environmental Affairs · Directorate: Biosecurity · Private Bag X 4390 · Cape Town · 8000 · 14 Loop Street · Cape Town 8001 · Tel: 021 441 2748 · Email: AISpermits@environment.gov.za


23. Destroy all packing material and wrapping by incineration or autoclaving.
24. Due to the invasive status of the plant material all experimental material/components shall be marked as potentially dangerous to the South African biodiversity.
25. No cultures, sub-cultures or specimens of the sample(s) may be given to any other person or be used for any work outside the facility.
26. No organisms shall be used in inoculation studies outside the facility without the written consent of the Issuing Authority.
27. Take all precautions at all times to prevent the spread and introduction of any organism(s), which may be present in/on the samples, into the RSA.
28. All samples and organisms isolated from the samples, shall be autoclaved or incinerated directly after completion of the research in the presence of the Environmental Management Inspector (EMI). Arrangements should be made with the EMI before the samples are destroyed at SKotze@environment.gov.za or AISCompliance@environment.gov.za
29. The Issuing Authority, (for attention: Mr. Khathutshelo Nelukalo : Email (AISpermits@environment.gov.za or Knelukalo@environment.gov.za) shall be notified immediately thereof in writing.
30. If any of the above-mentioned conditions are not complied with or are violated, the material shall be destroyed, at the permit holder's expense.
31. The permit holder must submit a report after completion of the research project together with the research findings.
32. The permit holder is responsible for the safety, conduct and training of all unnamed persons assisting with the work specified in the permit.
33. The permit holder is responsible for preventing their release to the environment.
34. All holding, transport, and culture systems must be designed, operated and maintained to prevent the escape of all life stages of nonnative aquatic species into waters of the South Africa. Water must be sterilised and disposed of after the teststo ensure no eggs, parasites or pathogens remain viable
35. The facility must have effective measures in place to prevent theft of listed species.
36. All research on *Eichhornia crassipes* shall be conducted in indoor facilities in containers or other confinement facilities designed to prevent escape and having no exterior water discharge or having a water discharge through a closed drain system that terminates in a dry-bed wastewater retention area with no public access.
37. Scheduled and unannounced inspections to ensure general security measures are followed may be conducted at any time during the permit period.

Page 3 of 3



000002037

Appendix A3.2: Permit for collection and transportation of water hyacinth


REPUBLIC OF SOUTH AFRICA

Department of Environmental Affairs
Issuing Authority, Department of Environmental Affairs, Directorate: Biosecurity Services, Private Bag X 4390, Cape Town, 8000
14 Loop Street, Cape Town, 8001. Tel: 021 441 2748, E-mail: AISpermits@environment.gov.za

**THIS PERMIT IS ISSUED IN TERMS OF CHAPTER 7 OF THE NATIONAL ENVIRONMENTAL
MANAGEMENT: BIODIVERSITY ACT, 2004 (ACT 10 OF 2004) (AS AMENDED) READ TOGETHER WITH
THE ALIEN AND INVASIVE SPECIES REGULATIONS, 2014 (AS AMENDED)**

PERMIT FOR RESTRICTED ACTIVITY OF ALIEN SPECIES OR LISTED INVASIVE SPECIES

Permit Holder Details:

Name: Rasheed Adegbola Adeleke
Address: 600 Belvedere Street, Arcadia, Pretoria, 0083

I/D/ Company Registration number: 7604026368185
Permit number : 5086577921
Date issued: 29/08/2017
Expiry date: 31/12/2018
Amount Paid: R0.00

Exporter/Supplier/Seller/Trader details:

Name: Hartbeespoort Dam
Address: Madibeng, North West

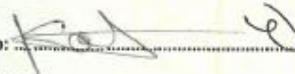
Premises where restricted activity will be carried out:
Physical Address: 600 Belvedere Street, Arcadia, Pretoria, Gauteng, 0083

Permission is hereby granted to the Permit Holder for the following: Convey under the attached permit conditions.

Species details

Scientific Name	Common Name	Quantity	Note
<i>Eichhornia crassipes</i>	Water hyacinth	± 1000	Plant species

Permit issued by: Khathutshelo Nelukalo
Place: Cape Town

Signature: 

Official stamp

DEPARTMENT OF ENVIRONMENTAL AFFAIRS REPUBLIC OF SOUTH AFRICA DIRECTORATE: BIO-SECURITY 29 AUG 2017 PRIVATE BAG X4390 CAPE TOWN 8000 ALIEN AND LISTED INVASIVE SPECIES: PERMIT
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000002038

PERMIT NUMBER: 5086577921



environmental affairs

Department:
Environmental Affairs
REPUBLIC OF SOUTH AFRICA

Issuing Authority: Department of Environmental Affairs · Directorate: Biosecurity · Private Bag X 4390 · Cape Town · 8000 · 14 Loop Street · Cape Town 8001 · Tel: 021 441 2748 · Email: AISSpermits@environment.gov.za

CONDITIONS FOR CONVEYING, MOVING OR OTHERWISE TRANSLOCATING *EICHHORNIA CRASSIPES* (WATER HYACINTH) IN TERMS OF THE REGULATIONS OF THE NATIONAL ENVIRONMENTAL MANAGEMENT: BIODIVERSITY ACT, 2004 (ACT NO.10 OF 2004).

1. This permit only authorises the conveying, moving or translocating of *EICHHORNIA CRASSIPES* (WATER HYACINTH) from HARTBEESPOORT DAM, MADIBENG, NORTH WEST to 600 BELVEDERE STREET, ARCADIA, PRETORIA, GAUTENG, 0083.
2. The transport authorisation is valid for 16 (sixteen) months from the date stipulated on your permit. The permit holder shall notify the Department of Environmental Affairs ("the Department") within 48 hours, prior to the movement of any live species authorised under this permit. Notifications shall be sent to Email address: AISSpermits@environment.gov.za or Telephone: 021 441 2748.
3. This permit is valid for multiple consignments.
4. It is the responsibility of the permit holder to ensure that the correct species is translocated, conveyed or moved. The permit holder is guilty of an offence should they fail to comply; this may result in criminal prosecution, the permit holder is referred to Regulation 35 of the Alien and Invasive Species (AIS) Regulations 2014, as amended. The permit holder shall keep the record of movement activities including date of movement conducted during the validity period of the permit; and in a format as requested by the Department.
5. The permit holder shall take all required steps to prevent or minimise the unauthorised spread of the species concerned.
6. Plants must be transported in appropriate packaging.



Appendix A3.3: Ethics approval letter

CAES RESEARCH ETHICS REVIEW COMMITTEE

National Health Research Ethics Council Registration no: REC-170616-051

Date: 26/09/2017

Ref #: **2016/CAES/102**

Name of applicant: **Ms R Makofane**

Student #: **58533613**

Dear Ms Makofane,

Decision: Ethics Approval

Proposal: Evaluation of water hyacinth suitability as a feedstock for biogas production

Supervisor: Dr R Adeleke

Qualification: Postgraduate degree

Thank you for the application for research ethics clearance by the CAES Research Ethics Review Committee for the above mentioned research. Approval is granted for the project.

Please note that the approval is valid for a one year period only. After one year the researcher is required to submit a progress report, upon which the ethics clearance may be renewed for another year.

Due date for progress report: 30 September 2018

The application was reviewed in compliance with the Unisa Policy on Research Ethics by the CAES Research Ethics Review Committee on 27 February 2017.

The proposed research may now commence with the proviso that:

- 1) The researcher/s will ensure that the research project adheres to the values and principles expressed in the UNISA Policy on Research Ethics.*
- 2) Any adverse circumstance arising in the undertaking of the research project that is relevant to the ethicality of the study, as well as changes in the methodology, should be communicated in writing to the CAES Research Ethics Review*



University of South Africa
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Committee. An amended application could be requested if there are substantial changes from the existing proposal, especially if those changes affect any of the study-related risks for the research participants.

- 3) The researcher will ensure that the research project adheres to any applicable national legislation, professional codes of conduct, institutional guidelines and scientific standards relevant to the specific field of study.

Note:

The reference number [top right corner of this communiqué] should be clearly indicated on all forms of communication [e.g. Webmail, E-mail messages, letters] with the intended research participants, as well as with the CAES RERC.

Kind regards,



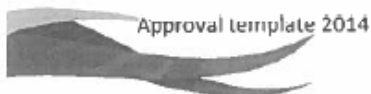
Signature

CAES RERC Chair: Prof EL Kempen



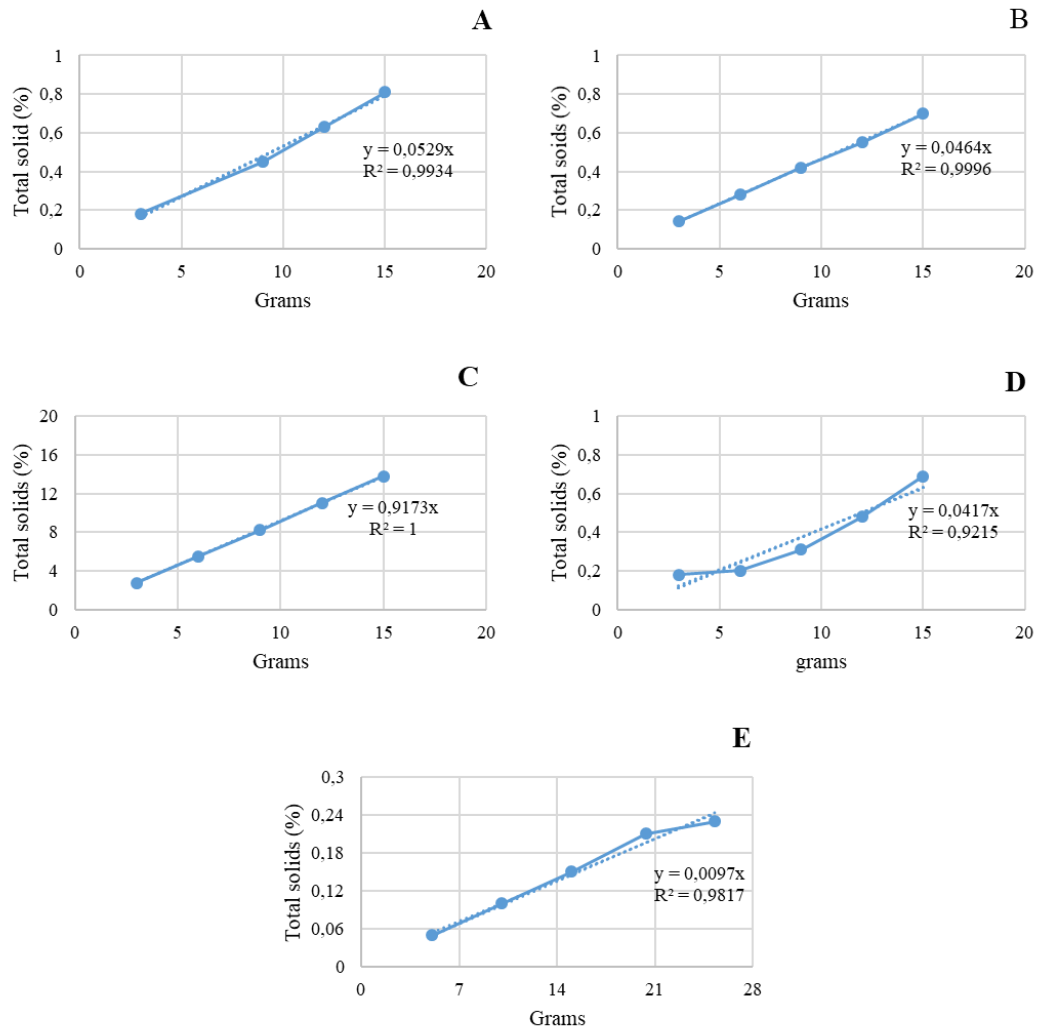
Signature

CAES Executive Dean: Prof MJ Linington



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Preller Street, Muckleneuk Ridge, City of Tshwane
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Telephone: +27 12 429 3111 Facsimile: +27 12 429 4150
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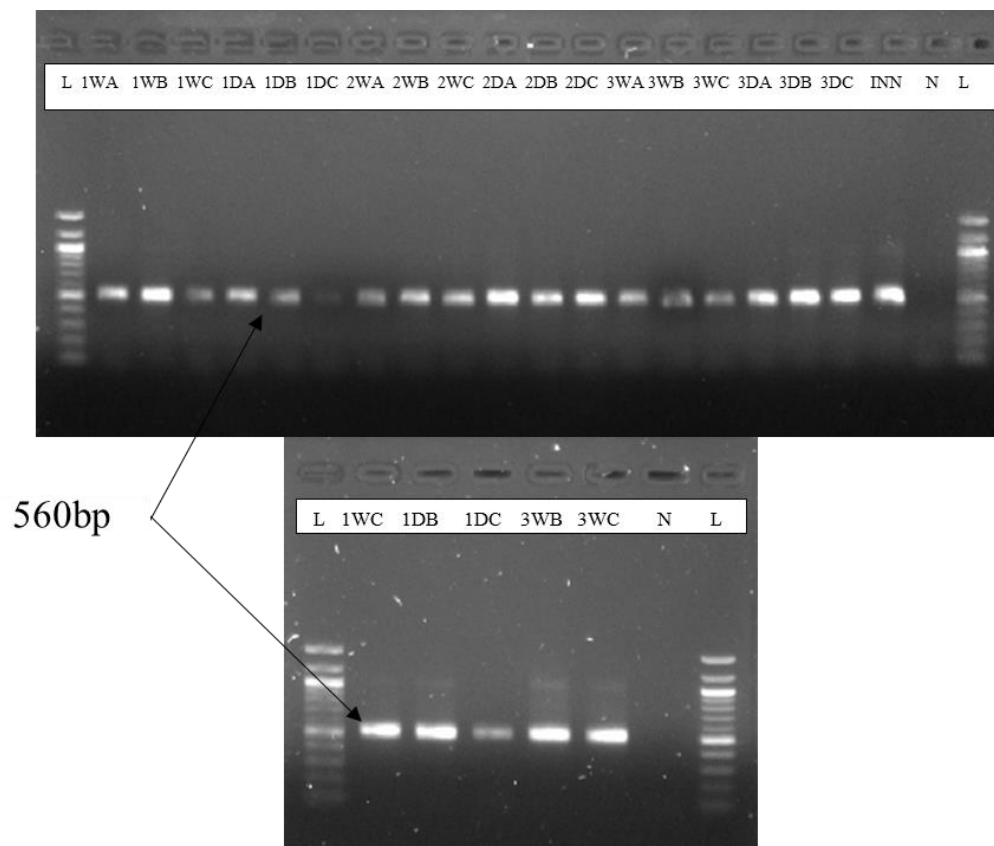
Appendix A3.4: Total solids calibration curves for all the pre-treatment methods and inoculum.
A-Hand cut, **B-** Homogenise, **C-** Sun dried, **D-**partial decomposition and **E-**Inoculum



Appendix A3.5: DNA concentration for the selected samples

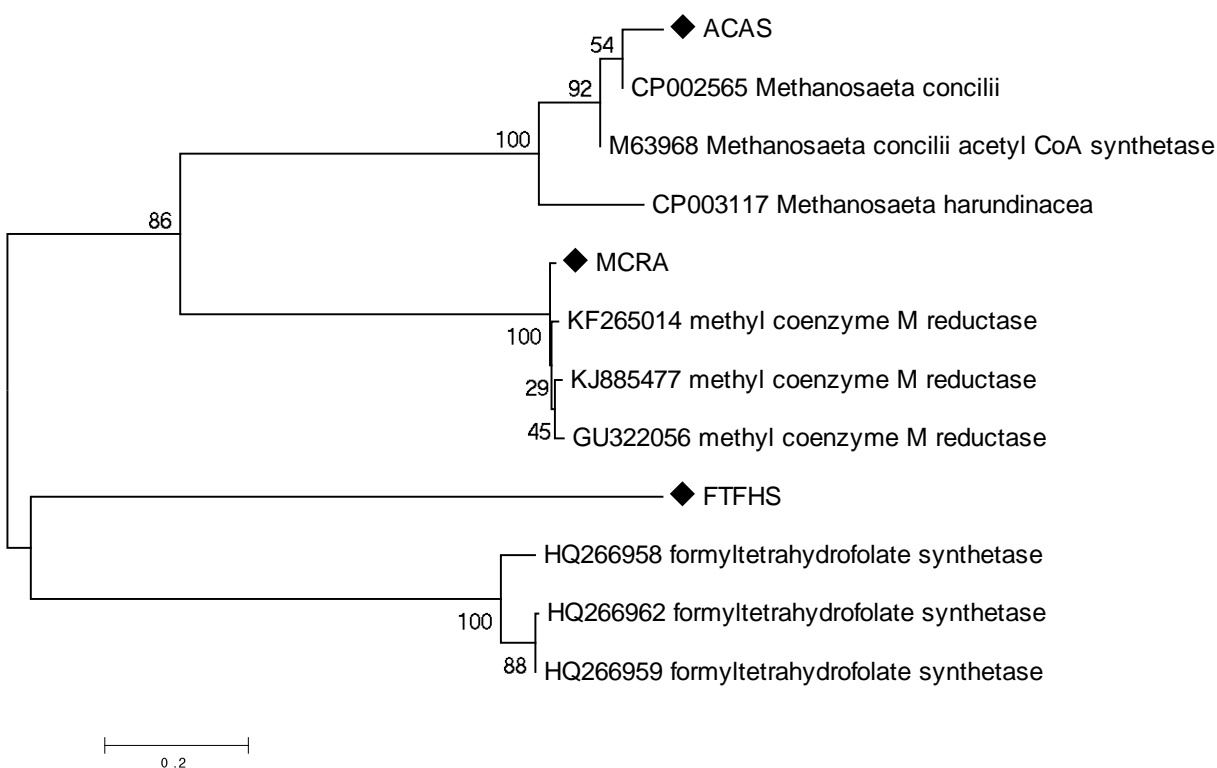
Sampling day	Concentration (µg/ml)	
	Mono-digestion	Co-digestion
20	3.98	2.98
29	7.14	2.60
38	2.28	4.94
41	3.18	7.38
50	6.26	3.70
59	5.62	6.10
62	8.24	7.78
71	7.06	3.74
80	7.68	8.40

Appendix A3.6: Amplification of the V3-V4 16S rRNA genes for illumina sequencing

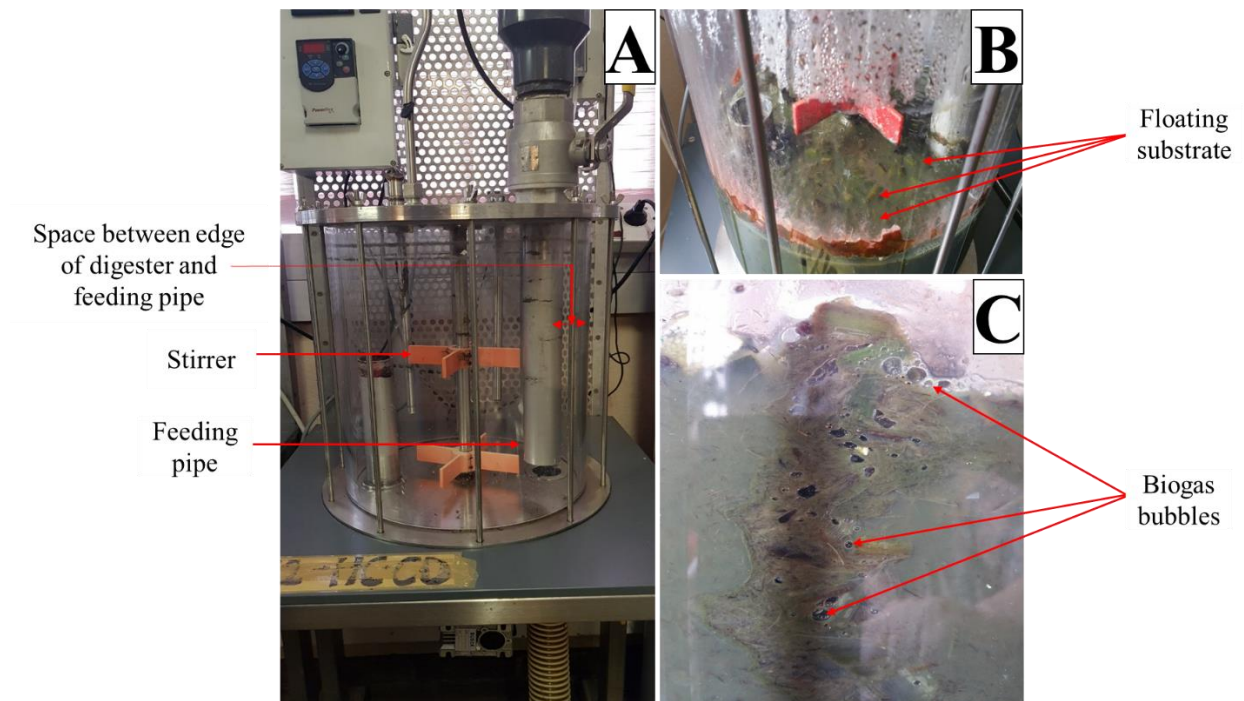


Appendix A4.1: Evolutionary relationships of taxa

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 3.28465195 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 478 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al, 2016).



Appendix A5.1: (A) Digester configuration and (B and C) challenges encountered during semi-continuous AD.



EVALUATION OF WATER HYACINTH (EICHHORNIA CRASSIPES) SUITABILITY AS FEEDSTOCK FOR BIOGAS PRODUCTION

by Rosina Makofane

Submission date: 27-Aug-2018 01:45PM (UTC+0200)

Submission ID: 993711071

File name: MSc_dissertation_2016.docx (15.73M)

Word count: 24499

Character count: 136134

EVALUATION OF WATER HYACINTH (EICHHORNIA CRASSIPES) SUITABILITY AS FEEDSTOCK FOR BIOGAS PRODUCTION

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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3	pdfs.semanticscholar.org Internet Source	<1 %
4	Shen, Zongzhuan, Dongsheng Wang, Yunze Ruan, Chao Xue, Jian Zhang, Rong Li, and Qirong Shen. "Deep 16S rRNA Pyrosequencing Reveals a Bacterial Community Associated with Banana Fusarium Wilt Disease	<1 %

Appendix A4: Language editing document of acknowledgement of their affiliation

Alexa Barnby
Language Specialist

Editing, copywriting, indexing, formatting, translation

BA Hons Translation Studies; APED (SATI) Accredited Professional Text Editor, SATI

Mobile: 071 872 1334

Tel: 012 361 6347

alexabamby@gmail.com

28 August 2018

To whom it may concern

This is to certify that I, Alexa Kirsten Barnby, an English editor accredited by the South African Translators' Institute, have edited the master's dissertation titled "Evaluation of water hyacinth (*Eichhornia crassipes*) suitability as feedstock for biogas production" by Rosina Makofane.

The onus is, however, on the author to make the changes and address the comments made.

